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# BACTERIOLOGICAL STUDY OF CARBOXYLMETHOXYLAMINE HEMIHYDROCHLORIDE<sup>1</sup>

CUTTING B FAVOUR

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A voluminous literature including a number of comprehensive reviews has appeared as a result of current interest in antibacterial agents (Waksman, 1941, Dubos, 1942, van Niel, 1943, Martin, 1944, Woolley, 1945-1946). Those substances which are active in the presence of serum and have a high therapeutic index have attracted the greatest attention because of their application to clinical medicine. Recently a search for preservatives for concentrated protein solutions has led to the study of carboxylmethoxylamine hemihydrochloride. This hydroxylamine, hereafter called compound I, in concentrated protein solutions has been found to be bacteriostatic against a wide variety of gram-negative as well as gram-positive microorganisms. Although its clinical toxicity, described elsewhere (Favour, 1947), largely limits it to *in vitro* work, it nevertheless has many practical uses. Accordingly, its antibacterial properties are presented as the subject of this report.

## EXPERIMENTAL RESULTS

*Physical properties* Compound I is a slightly hygroscopic, white, crystalline powder which is readily soluble in the usual bacteriological culture media and in concentrated protein solutions. In aqueous solution it is an acid. It retains its antibiotic powers when buffered in neutral or alkaline solutions, after filtration or autoclaving, and during many months of storage in solution.

*Procedures* Using the pour plate method and cultures in the logarithmic growth phase, a number of bacterial species were studied (table 1). Much of the detailed work was done with a strain of *Escherichia coli* the susceptibility of which to the compound was intermediate and constant, permitting its use for bioassay of stored and otherwise treated solutions.

In the course of the study we were supplied with two so-called "room temperature" coliform organisms which had been recovered from human albumin during processing. For some time they escaped detection by the usual cultural routines because they were inhibited or killed at 37 C but grew on ordinary media at 23 C. One of these was spontaneously resistant to the compound. The "indicator organism" (table 1) was recovered from a bottle of bromthymol blue. In the species studied there was no relation between relative resistance and ability to ferment sugars (see comments on mode of action). *Brucella*, for example, a non-carbohydrate fermenter, was among the most susceptible organisms studied.

<sup>1</sup> This work has been carried out under a contract, recommended by the Committee of Medical Research, between the Office of Scientific Research and Development and Harvard University.

**Drug resistance** A single colony of the stock *E coli* strain was subcultured in broth with an inoculum of 10,000 organisms per ml and transferred to tubes of broth containing ascending concentrations of the compound The first tube

TABLE 1

Approximate amount of compound I required to inhibit 1,000 organisms per ml in broth and in albumin

and in albumin

	TRYPTIC DIGEST BROTH	25% HUMAN ALBUMIN
	mg %	mg %
<i>Escherichia coli</i>	20	20
<i>Eberthella typhosa</i>	30	20
<i>Salmonella paratyphi</i>	20	20
<i>Salmonella schottmuelleri</i>	20	20
<i>Salmonella choleraesuis</i>	20	20
<i>Shigella paradysenteriae</i>	20	20
<i>Shigella sonnei</i>	20	20
<i>Proteus vulgaris</i>	50	30
<i>Proteus O<sub>1</sub>K</i>	20	20
<i>Proteus O<sub>1</sub>19</i>	20	20
<i>Proteus O<sub>2</sub></i>	20	20
<i>Pseudomonas aeruginosa</i>	50	20
<i>Vibrio metchnikovi</i>	10	10
<i>Brucella abortus</i>	30	30
<i>Corynebacterium diphtheriae</i>	10	10
<i>Corynebacterium hofmanni</i>	10	10
<i>Bacillus subtilis</i>	10	10
<i>Bacillus anthracis</i>	10	10
<i>Staphylococcus aureus</i>	10	10
<i>Staphylococcus albus</i>	10	10
<i>Streptococcus pyogenes</i>	10	10
<i>Streptococcus viridans</i>	10	10
<i>Diplococcus pneumoniae</i> type III	10	10
<i>Mycobacterium tuberculosis</i>	Albumin-oleic acid medium with 20 mg % compound	
<b>Note</b> Spontaneously resistant organisms follow		
"Room temperature organism" 1	1,000	?
"Room temperature organism" 2	100	30
"Indicator organism"	150	?
Yeasts	1,000	
Molds	Grew on plates containing 50 mg %	
Viruses	See discussion	

showing visible growth was similarly subcultured in a second series of ascending concentrations of the compound and the process carried through 18 such subcultures In the first subculture the *E coli* resistance jumped from 20 to 125 mg per cent, at which it remained thereafter Coincidentally, the organisms

which did grow were small, grew very slowly, and on the first subculture on drug-free medium usually failed to take up the red dye from McConkey's medium or to form the usual surface colonies. The first or second subculture on normal medium restored cultural morphology and the original drug sensitivity. Fermentation studies on organisms taken from the small phase were the same as those from normal *E. coli*, perhaps because of the rapid return to their previous growth characteristics.

Sporeforming organisms (*Bacillus subtilis* and *Bacillus anthracis*) in the vegetative phase behaved like *E. coli*. Inhibited cultures, however, could be subcultured even when exposed to 100 mg per cent compound for 13 days. Ten mg per cent compound was inactive against spores even when the temperature was held at 58 C for 2 hours.

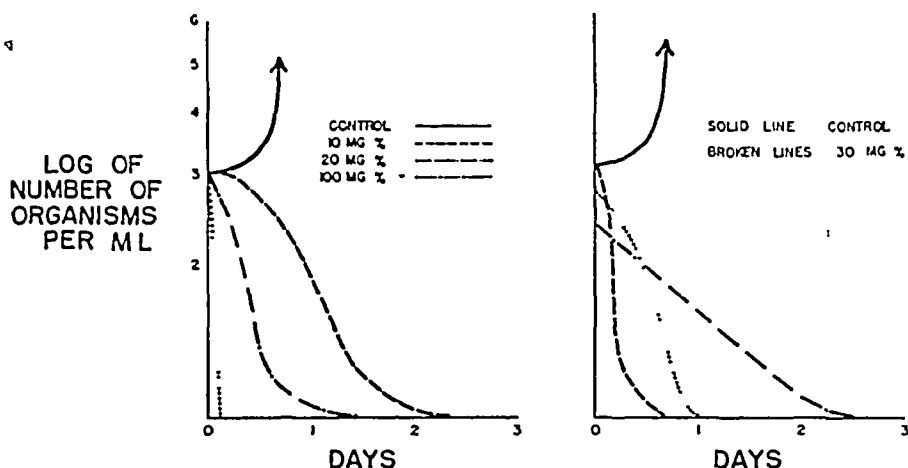


FIG. 1. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE IN 25 PER CENT ALBUMIN ON *E. COLI*.

Sporeforming organisms (*Bacillus subtilis* and *Bacillus anthracis*) in the vegetative phase behaved like *E. coli*. Inhibited cultures, however, could be subcultured even when exposed to 100 mg per cent compound for 13 days. Ten mg per cent compound was inactive against spores even when the temperature was held at 58 C for 2 hours.

**Drug concentration.** With the use of small inocula of *E. coli* in a relatively poor medium (25 per cent human albumin) a difference between bacteriostatic and bactericidal drug concentrations can be seen (figure 1). The difference between these concentrations is greater for a relatively resistant organism such as *Proteus vulgaris* (figure 2) and less for a susceptible organism such as the hemolytic streptococcus (figure 3).

**Size of inocula.** From 2,000 to 10,000 organisms per ml, depending on other factors, are prevented from growing, but the larger inocula are not inhibited by proportional increases in drug concentration unless the medium is very poor in growth factors (figure 4). On the other hand, very small inocula in a poor me-

drum may survive longer than a larger inoculum in the same drug concentration (figure 1) Likewise a given inoculum in a poor medium may survive longer than a larger inoculum in the same drug concentration (figure 6) In addition, a

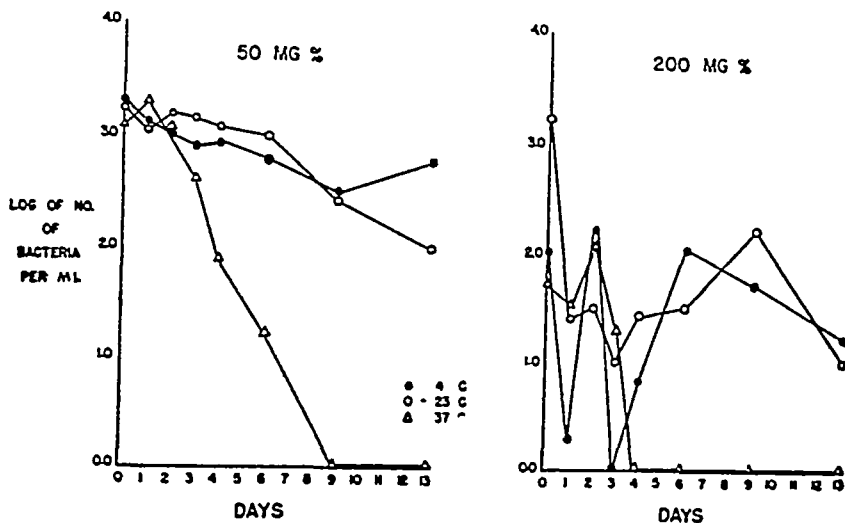


FIG 2 EFFECT OF CARBOXYLMETHOXYLAMINE ON *PROTEUS VULGARIS* TRYPTIC DIGEST BROTH

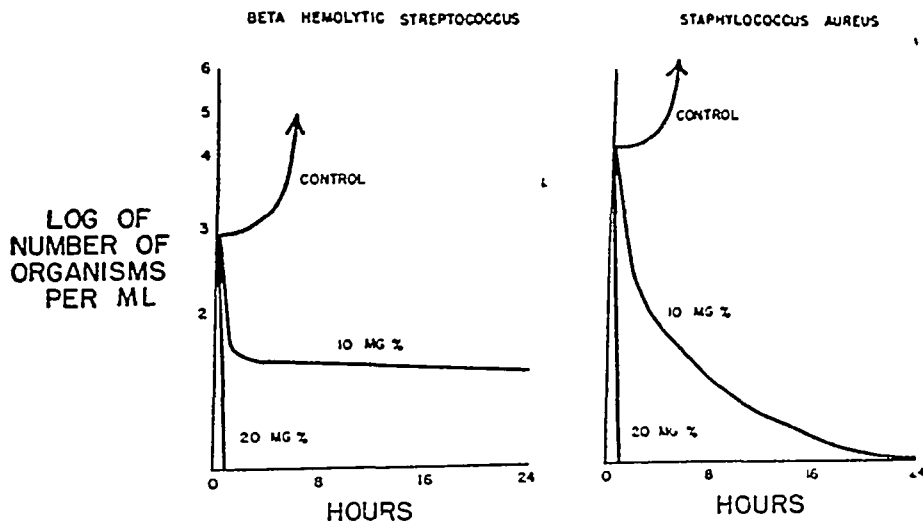


FIG 3 IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE IN 25 PER CENT ALBUMIN

given inoculum in saline will survive longer at an intermediate drug concentration than at a high or low concentration (figure 5) and a moderate inoculum in water will survive longer with less than the optimal amounts of growth factors (figure 6)

**Blocking antibiotic effect** When sodium pyruvate and compound I, mg for mg, are added to a medium, the antibiotic effect of the drug is completely

blocked Acetone in somewhat higher concentrations also will block the action of the compound (see discussion) Thioglycolate (Brewer's medium) with or without methylene blue completely blocks the antibiotic action of the compound

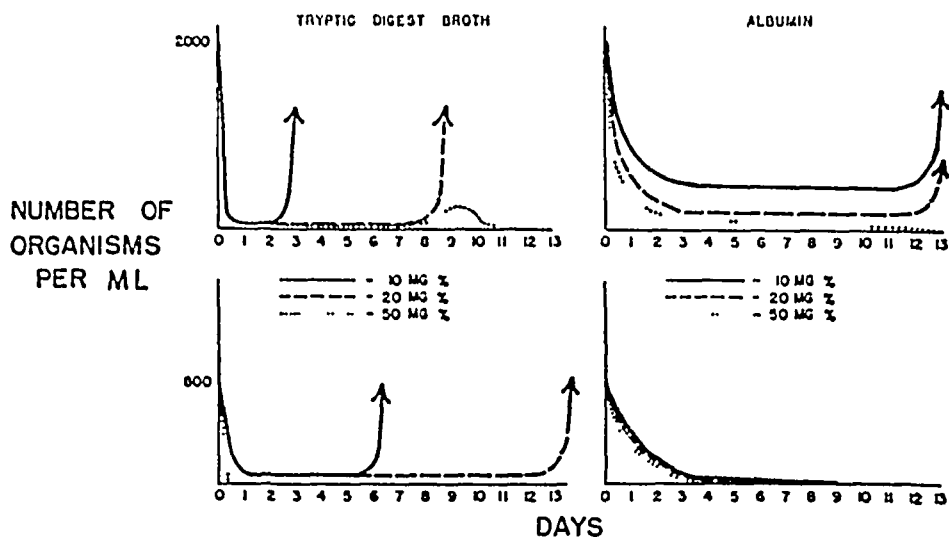


FIG 4 IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE ON *E. COLI*

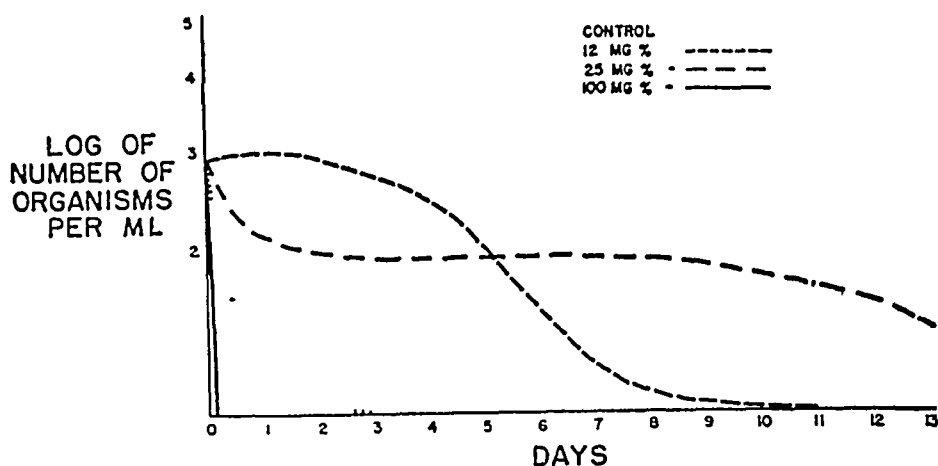


FIG 5 IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE ON WASHED *E. COLI* IN 0.85 PER CENT SODIUM CHLORIDE

*Efforts to potentiate antibiotic effect* Because of the carbohydrate cycle suggested for tissues by Toennissen and Brinkmann (1930), an effort was made to inhibit succinic dehydrogenase at the same time compound I was present, using iodoacetic acid (Oxford, 1942) and malonate. With *E. coli* in tryptic digest broth, 400 organisms per ml, and varying concentrations of compound I from 2 to 20 mg per cent, it was found that similar concentrations of iodine-free iodo-

acetic acid, equal concentrations of sodium malonate, or the combination of the two did not enhance the effect of the compound

Gamma globulin solutions (fractions II and III), 18 per cent, have a natural antibacterial power which varies with the lot and the bacterial species Growth

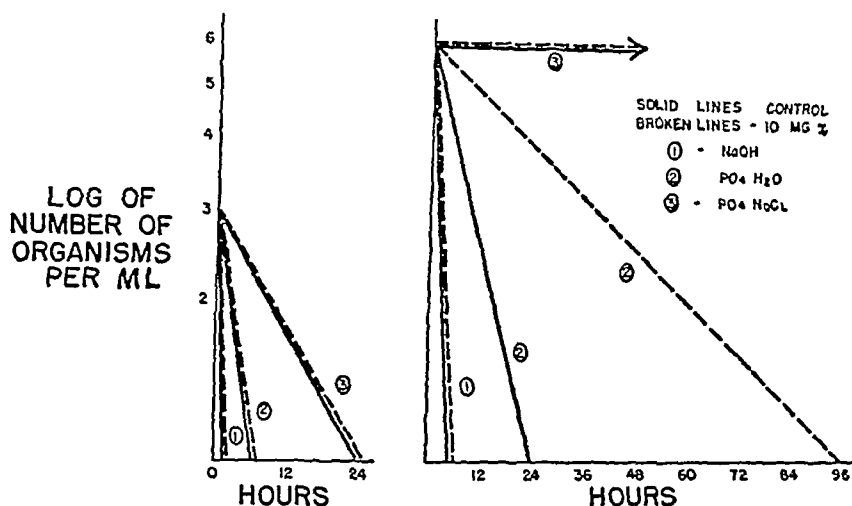


FIG 6 IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE ON WASHED *E. typhi*

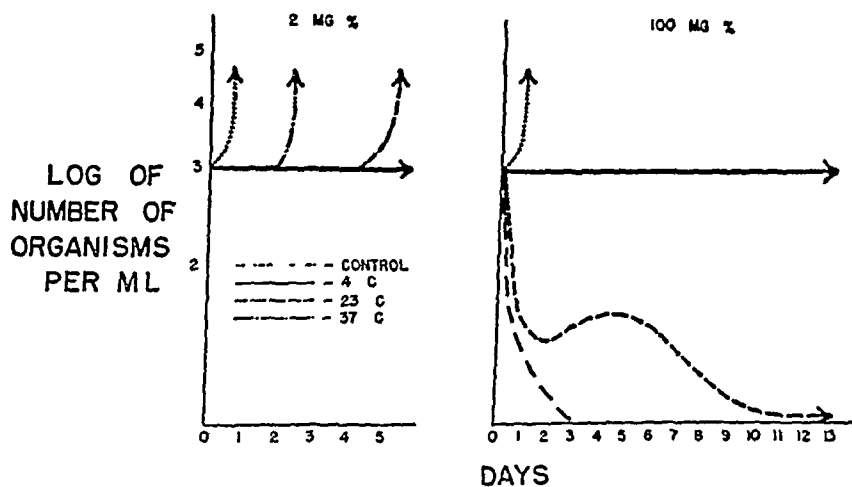


FIG 7 IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE IN 25 PER CENT ALBUMIN ON *E. coli*

inhibition or bacterial death takes place during 3 to 5 days at 37 C Thereafter, bacteria grow luxuriantly in globulin unless inhibited by a preservative

*Time of drug exposure* In time bacteriostatic concentrations of compound I are bactericidal but not necessarily so (figure 6) Slides of cultures taken in the bacteriostatic phase show pleomorphism, gigantism, and failure of complete fission Pour plate studies in this interval show no increase in the number of viable organisms (See, also, comments on efforts to induce drug resistance)

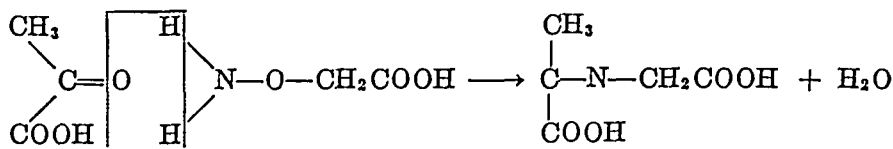
*Temperature* Unlike some metabolic inhibitors, such as mercury derivatives, compound I is relatively inactive at low temperatures (figure 7)

*Media* A small inoculum is more readily suppressed and killed in a poor medium than in a medium with many growth factors (figure 4). Nonnutrient medium does not necessarily potentiate compound I on proportionately larger inocula (figure 6). Even the presence of the small amount of phosphate ions used to adjust the pH to neutral may alter the survival time of bacteria (figure 6). On the other hand, twice molar glucose or equimolar lactate in broth, each indirect sources of pyruvate in the body, do not alter the antibacterial properties of the compound.

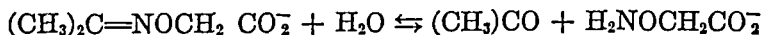
#### DISCUSSION

The present studies on carboxylmethoxylamine hemihydrochloride indicate that it belongs to the group of metabolic inhibitors. Like the arsenicals it has a low therapeutic index, and like the sulfonamides it is bacteriostatic and must be used in relatively high concentrations. Like penicillin (Oxford, 1942) it prevents cell division and promotes bacterial pleomorphism. And finally, like the salts of heavy metals, its wide range of biological toxicity is matched by its antibacterial effects on a broad range of bacterial species. Unlike most such agents, its effects are limited to small inocula. Although it is too toxic for use as a preservative in large-volume, parenteral solutions, it has certain special uses in which other substances are ineffective. Among these is the preservation of blood-typing serum, bacterial vaccines, and other preparations with a high protein content. No doubt, in time still other uses will be found. An unexplored field is virus work. The influenza virus, PR8 strain, is not inhibited by bacteriostatic concentrations of the compound (Stanley, 1947).

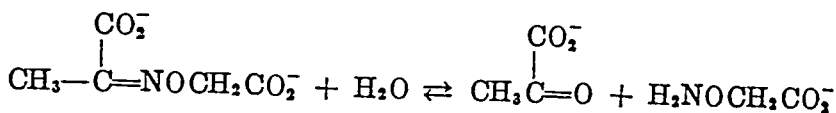
Another subject for further study is the mechanism of action of the compound on both tissues and on bacteria. Previous reports on the antibiotic effect of hydroxylamines have indicated their wide range of species toxicity (Mayer and Oechsle, 1937, Burton *et al.*, 1940). Clarke and associates (1947) demonstrated the ability of pyruvate to block the *in vivo* antibiotic effect of compound I on cellular metabolism. From these observations it would seem that carboxylmethoxylamine is a metabolic inhibitor by virtue of its ability to combine with alpha ketones in living cells, the most important of these being pyruvate.



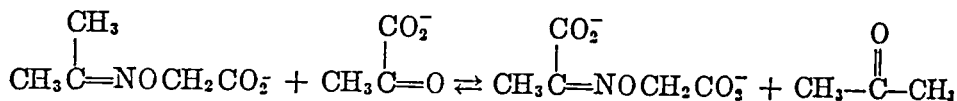
Acetone has a similar blocking action. This, Clark feels, is due to the fact that oximes undergo appreciable hydrolysis.



The pyruvic compound undoubtedly acts similarly, though the composition at equilibrium may not be the same



If a second aldehyde or ketone is added, the equilibrium is disturbed with the regeneration of some of the original carbonyl compound and the formation of a new carboxymethoxime. For example, if acetone carboxymethoxime be added in excess to pyruvate, an appreciable proportion of the pyruvate will be removed from the system as its carboxymethoxime and a corresponding amount of acetone will be liberated



This would explain why acetone carboxymethoxime possesses vestigial anti-bacterial properties and why the carboxymethoxime of pyruvate, which of course is incapable of tying up any pyruvate it may encounter, is quite inactive. Aldehydes and alpha-keto acids other than pyruvic acid should act in a similar fashion

#### SUMMARY

Carboxymethoxylamine hemihydrochloride belongs to the group of metabolic inhibitors. It is bacteriostatic in small concentrations (30 mg per cent) and bactericidal in large concentrations (100 to 1,000 mg per cent).

Its antibiotic activity is presumably due to its ability to combine with alpha ketones, the most important in living organisms being pyruvate. Its toxicity *in vitro* is counteracted by pyruvate, acetone, and thioglycolate, but not by lactate, glucose, anaerobic conditions, methylene blue or its leuco form, cysteine aerobically or anaerobically, iodoacetic acid, or malonate.

Inocula (1,000 organisms or less) of a wide variety of common gram-negative and gram-positive organisms are inhibited. Occasional spontaneously resistant coliform organisms have been encountered. Viruses, yeasts, and fungi are not affected by the compound. Only the vegetative forms of sporeforming bacteria are affected.

The compound is too toxic for large-volume parenteral use. However, it can be used as a preservative for concentrated protein solutions such as typing sera or in vaccines as a supplement to routine aseptic techniques in order to combat small chance contaminations.

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# THE INHIBITORY EFFECT OF LIPASE ON BACTERIAL GROWTH IN MEDIA CONTAINING FATTY ACID ESTERS

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It has been reported from this laboratory that serum albumin is essential for the initiation of growth by small inocula of tubercle bacilli in a liquid medium containing "tween 80"<sup>2</sup> which promotes submerged growth of these organisms (Dubos, 1945, Dubos and Davis, 1946). The albumin acts as a protective rather than a nutritive growth factor, binding traces of unesterified fatty acid which inhibit growth of small inocula (Davis and Dubos, 1947).

The commercial bovine serum albumin, however, also makes the medium somewhat unstable, which is particularly important because tubercle bacilli grow so slowly. Inocula containing only small numbers of bacilli (ca. 10 to 20) require a period of cultivation of 2 weeks to attain visible growth, yet after sterile incubation of the tween-albumin medium at 37 C for 2 weeks, it was unable to support growth of even large inocula. It appeared possible that in the race between bacterial multiplication and spontaneous deterioration of the medium even smaller inocula might be prevented from surviving. This prediction could be confirmed after the deterioration was traced to hydrolysis of tween 80 by lipase in the commercial albumin, which released free fatty acid in bacteriostatic quantities exceeding the binding capacity of the albumin. Elimination of the lipase permitted cultivation of even smaller inocula, such as 2 or 3 bacteria.

Lipolysis was also found to account for the bacteriostatic effect of several materials of biological origin (sera, bacterial culture filtrates), observed only in media containing tween 80. This effect calls attention to precautions necessary in using media containing tween.

The results of the experiments involving lipase, which have received a preliminary report (Davis and Dubos, 1946), are presented in this paper. Although the bacteriological work was confined to human tubercle bacilli, the principles evolved are applicable to the cultivation, in the presence of esters, of any bacteria that are sensitive to free fatty acid.

## EXPERIMENTAL METHODS AND RESULTS

The methods of cultivation have been reported elsewhere (Dubos and Davis, 1946). The semisynthetic liquid medium contained a salt mixture buffered at pH 7.0, enzymatic casein hydrolyzate, yeast autolyzate, glucose, 0.05 per cent tween 80, and 0.1 per cent bovine serum albumin (fraction V, Armour), unless

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<sup>2</sup> Trade name of a standardized commercial polyoxyethylene derivative of sorbitan monooleate

otherwise specified. It was distributed in amounts of 5 ml in wide (25-mm) metal-capped test tubes. The inoculum was a standard laboratory strain of virulent human tubercle bacilli (H37Rv) that had been grown for many passages in the tween-albumin medium. The inocula are designated in the tables in terms of volume of a 7- to 10-day-old culture in the same medium, containing approximately 1 mg moist weight of organisms per ml, in one experiment (table 6) accurately measured inocula were used. Growth is recorded in the tables in terms of a visual estimate ranging from 0 (no visible growth) to 4 (full growth, approximately 2 mg moist weight per ml).

In experiments involving prolonged storage of media at 37 C, evaporation was prevented by parafilm covering the cotton plugs.

Chemical estimations of lipase activity were based on a method of extracting and titrating fatty acid that was developed for this purpose (Davis, 1947a). A somewhat similar method of measuring lipase activity was developed simultaneously by Archibald (1946).

#### *Deterioration of Tween-Albumin Medium*

It was repeatedly observed that sterile incubation for 14 to 21 days at 37 C destroyed the capacity of the medium to support the growth of even large inocula of tubercle bacilli. Since the minimal growth requirements of tubercle bacilli are very simple, the deterioration of the medium during the incubation seemed likely to have been caused by formation of a bacteriostatic product rather than by destruction of a growth factor. The medium could be stored for much longer periods in the refrigerator without deterioration.

To detect the unstable component a group of partial media were prepared, each lacking in one of the major components. These were stored under sterile conditions for 21 days at 37 C. Portions of sterile concentrated solutions of each of the components were simultaneously stored in the refrigerator. At the end of the period of preliminary incubation the absent component was aseptically added to each incomplete batch of medium, so that the final composition of the various batches was identical except for changes that had taken place during storage. These media were then distributed in 5-ml volumes, inocula of various sizes were added, and the tubes were further incubated.

The results, which are presented in table 1, show that two components of the medium were involved rather than one, deterioration took place only if serum albumin and tween 80 were incubated simultaneously. Since other experiments had shown that tween 80 contains somewhat bacteriostatic concentrations of free oleic acid (Davis, 1947a) and, further, hydrolyzes slowly in aqueous solution (Davis, 1947b), and since serum is known to contain lipase, it appeared very likely that the commercial albumin was contaminated by traces of lipase that accelerated the hydrolysis of the tween, so that after a sufficient period of incubation the binding capacity of the albumin for fatty acid was exceeded.

This interpretation was strengthened by the observations (table 2) that further addition of albumin, at the end of the preliminary incubation, overcame the toxicity of deteriorated tween-albumin medium and that commercial crystalline

TABLE 1

*Deterioration of incomplete media during storage*

NATURE OF MEDIUM DURING STORAGE	TEMPERATURE OF STORAGE (C)	INOCULUM (MG MOIST WEIGHT)				
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
		Growth*				
Complete	37	4	$\frac{1}{2}$	0	0	0
Glucose absent	37	4	1	0	0	0
Casein hydrolyzate absent	37	1	0	0	0	0
Mineral mixture absent	37	1	1	0	0	0
Albumin absent	37	4	3 $\frac{1}{2}$	2 $\frac{1}{2}$	$\frac{1}{2}$	$\pm$
Tween 80 absent	37	1	3	1 $\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$
Complete	5	4	3 $\frac{1}{2}$	2 $\frac{1}{2}$	1 $\frac{1}{2}$	$\frac{1}{2}$
Complete, freshly prepared		4	3	2 $\frac{1}{2}$	1	$\frac{1}{2}$

Incomplete media were stored for 21 days at 37 C. The deficiencies were then restored and the complete media inoculated in volumes of 5 ml. Complete media stored meanwhile in the refrigerator, or freshly prepared, were inoculated simultaneously.

\* Growth recorded at 12 days

TABLE 2

*Restoration of medium deteriorated during storage: stability of medium containing crystalline albumin*

MODIFICATION OF MEDIUM	TEMPERATURE OF STORAGE (C)	INOCULUM (MG MOIST WEIGHT)				
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
		Growth				
None	5	4	4	3	2 $\frac{1}{2}$	0
Crystalline albumin	5	4	3 $\frac{1}{2}$	3	2 $\frac{1}{2}$	2
Tween 60	5	4	4	2 $\frac{1}{2}$	$\frac{1}{2}$	0
None	37	4	0	0	0	0
Tween 60	37	0	0	0	0	0
Crystalline albumin	37	4	3 $\frac{1}{2}$	3	2	2
Additional fract V	37	4	3 $\frac{1}{2}$	3	2 $\frac{1}{2}$	1 $\frac{1}{2}$

Complete media, containing 0.05 per cent tween 80 and 0.1 per cent fraction V, as well as media modified as indicated (tween 60, a stearic ester, substituted for tween 80, an oleic ester, crystalline albumin substituted for fraction V), were stored at 37 C and 5 C for 18 days. To one lot of unmodified medium additional fraction V (0.1 per cent) was added at the end of the period of storage. Volumes of 5 ml were inoculated and growth was recorded after 14 days of incubation.

The medium containing tween 60 and albumin which had been stored at 37 C showed a slight precipitate in all tubes following the second period of incubation, as well as complete absence of growth. At this stage of investigation this was the observation which led to the recognition of hydrolysis of the tweens by albumin, for oleic acid is more soluble than stearic acid, and hence there had been no visible evidence of hydrolysis of tween 80.

serum albumin, which is purer than fraction V, did not cause deterioration during storage

### *Presence of Lipase in Commercial Serum Albumin*

Direct proof of this explanation of the instability of the tween-albumin medium required demonstration of lipase in the serum albumin. All efforts to demonstrate lipase by the usual method of measuring changes in titrable acidity were unsuccessful even following incubation of tween solutions with albumin for as much as several days, the buffering power of the albumin concealed any lipolysis that might have taken place. Similar negative experiments were obtained with a classical lipase substrate, tributyrin. It was therefore necessary to develop a

TABLE 3  
*Measurement of lipase in albumin*

ADDITION TO SUBSTRATE	TITER N/50 NaOH AT TIME			
	0	24 hr	48 hr	72 hr
Control—none	0 13	0 14	0 13	0 16
Albumin (fract V, unheated)	0 17	0 26	0 38	0 55
Albumin + 0.03% NaF	0 17	0 20	0 21	0 215
Albumin heated at 56 C in 1% NaCl, pH 5.6	0 15	0 14	0 14	0 135

The substrate consisted of 0.5 per cent tween 80 in 0.02 M phosphate buffer at pH 7.0, or in the mineral mixture (pH 7.0) used in the bacteriological medium, similar results were obtained in either medium. A series of tubes containing 10 ml of substrate and albumin (1 ml 5 per cent) and fluoride as indicated, were incubated at 37 C, with the addition of a crystal of thymol to inhibit bacterial growth. At 24 hour intervals a tube from each group was extracted and titrated for ether-soluble acid. The blank values are high because the tween was not freed of fatty acid before incubation.

more sensitive method, involving extraction of the free fatty acid from the substrate-albumin mixture<sup>3</sup> (Davis, 1947a)

Table 3 shows that the commercial serum albumin hydrolyzes tween 80. It further shows, in anticipation of the following section, that the lipolytic activity is destroyed by heating at 56 C for 30 minutes, indicating an enzymatic activity. The amount of lipase is so small that incubation for several days was necessary for its measurement.<sup>4</sup>

<sup>3</sup> Tween 80 was used as the substrate for measuring lipase since it was the ester of chief biological interest. A closely related ester was found to be hydrolyzed by albumin somewhat more rapidly and hence to serve as a more sensitive substrate. This product is designated by the Atlas Powder Company as G-2144, it consists of the polyoxyethylene derivative of oleic acid and differs from tween 80 in the absence of sorbitan.

<sup>4</sup> It is possible that the lipase activity might have been more simply measured in a Warburg respirometer, but it may be calculated from the data of table 3 that the addition of as large an amount of albumin as 0.2 ml of 5 per cent solution to a respirometer vessel would have resulted in the evolution of only 4 mm<sup>3</sup> of CO<sub>2</sub> per hour, assuming that the buffering capacity of the protein would not have interfered with the gaseous equilibration.

*Elimination of Lipase from Albumin*

It was found possible to eliminate the lipase activity by any of three methods (a) heating the albumin sufficiently to destroy the lipase, (b) using purer albumin that is not contaminated by lipase, or (c) using fluoride as a lipase inhibitor

*Heated albumin* The capacity of serum to bind fatty acid is dependent upon the essentially native configuration of the protein molecule, being destroyed by enzymatic digestion or by heating to 100 C even under certain conditions that avoid coagulation of the denatured protein (Davis and Dubos, 1947) Fortunately, however, the lipase proved to be more thermolabile than the fatty-acid-binding capacity of the albumin The lipase effect was completely destroyed by heating 5 per cent albumin solution at 56 C for 30 minutes In order to avoid coagulation of the albumin at this temperature, it is necessary either to neutralize the albumin solution (which has an initial pH of approximately 5.6) or to add 1 or 2 per cent NaCl The higher concentration of NaCl is safer, as 1 per cent is not sufficient to prevent coagulation if the temperature rises slightly above 56 C Since the heated albumin solution tends to clog the filters by which it is sterilized, it is advisable to filter before heating <sup>5</sup>

Since the coagulability of albumin varies markedly with salt concentration and pH, it was necessary to determine whether the conditions which reduced the danger of coagulation of the albumin might not also reduce the destruction of lipase Table 4 shows that albumin heated at 56 C under any of the conditions described above causes no deterioration of the medium following incubation for 20 days, in contrast to unheated albumin The possibility of slight differences in residual lipolytic activity of these albumins, of course, is not ruled out by this bacteriological evidence

Table 5 presents evidence that the deteriorated medium which had been incubated with unheated albumin contained more free fatty acid than that which had been stored in the refrigerator or incubated with heated albumin Since albumin can protect tubercle bacilli against only 1 to 2 per cent of its weight of oleic acid (Davis and Dubos, 1947), the amount of fatty acid freed in the deteriorated medium (0.002 per cent) is sufficient to saturate the protective capacity of the albumin (0.1 per cent) Since some free oleic acid is present in the medium in addition to that specifically hydrolyzed at 37 C, and is further released during incubation, the failure of the medium to support growth is quantitatively accounted for by its content of oleic acid

*Crystalline serum albumin* Crystalline bovine serum albumin, which is purer than fraction V, is also commercially available (Armour) This material contains no significant amounts of lipase, the medium containing crystalline albumin

<sup>5</sup> Salt was observed to have a dual effect on the thermal stability of albumin Whereas NaCl protected the albumin at pH 5.6 from coagulation when heated to 56 C, the opposite effect was observed at pH 7.0, at which a solution of albumin without added NaCl could be boiled without coagulation, but a solution with added NaCl (0.5 per cent or more) coagulated when boiled The boiled, uncoagulated albumin, however, was denatured, not only had it lost its power to bind oleic acid (Davis and Dubos, 1947), but it precipitated on addition of acetic acid

rather than fraction V showed no deterioration after incubation for 18 days, as was shown in table 2

*Fluoride* Fluoride ion is a well-known inhibitor of lipase (Loevenhart and Pearce, 1907) It is also a bacteriostatic agent, but fortunately the lipase in serum albumin was effectively inhibited by a concentration of NaF (0.01 per cent) that did not interfere with growth of tubercle bacilli in this medium. The absence of bacteriostasis may be related to the high concentration of Mg in this medium (0.1 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), since the inhibitory effect of fluoride on

TABLE 4  
*Destruction of lipase in heated albumin (bacteriological evidence)*

TYPE OF ALBUMIN	TEMPERATURE OF STORAGE	INOCULUM (MG MOIST WEIGHT)			
		$10^{-2}$	$10^{-4}$	$10^{-6}$	$10^{-8}$
		Growth*			
Unheated, 2% NaCl (clear)	37	±	0	0	0
	5	3	2	1	$\frac{1}{2}$
Heated, 2% NaCl (sl opalescent)	37	3	2	1	$\frac{1}{2}$
	5	3	2	1	$\frac{1}{2}$
Heated, 1% NaCl (sl opalescent)	37	3	2	1	$\frac{1}{2}$
	5	3	2	1	$\frac{1}{2}$
Heated, neutral, 2% NaCl (clear)	37	3	2	1	$\frac{1}{2}$
	5	3	2	1	$\frac{1}{2}$
Heated, neutral, 0 NaCl (clear)	37	3	2	1	$\frac{1}{2}$
	5	3	2	1	$\frac{1}{2}$

Sterile bovine serum albumin (fraction V) in a concentration of 5 per cent in various solutions was heated at 56 C for 30 minutes. Lots of medium containing 0.05 per cent tween 80 and 0.1 per cent of these various albumins, as well as unheated albumin, were stored for 20 days at 37 C and at 5 C, they were then distributed in 5 ml volumes and inoculated with dilutions of tubercle bacilli.

\* Growth recorded at 9 days

some enzymes has been shown to be due to the formation of magnesium fluorophosphate complexes (Warburg and Christian, 1942)

It is shown in table 3 that the addition of 0.03 per cent NaF essentially completely inhibits the lipase of fraction V. Evidence of the effectiveness of a bacteriological medium containing fluoride is presented in table 6

#### *Effect of Elimination of Lipase on Bacterial Growth*

Table 6 also shows that elimination of lipase activity by any one of the three methods described above permitted growth of tubercle bacilli following inoculation with one further 10-fold serial dilution ( $10^{-8}$  mg moist weight) than was possible with unheated fraction V. Other similar comparative experiments showed a difference of two serial dilutions.



To find how few bacteria constitute the minimum effective inoculum, an experiment was carried out with inoculation by means of dilutions of droplets

TABLE 5  
*Destruction of lipase in heated albumin (chemical evidence)*

TYPE OF ALBUMIN	TEMPERATURE OF STORAGE (C)	TITER N/50	DIFFERENCE
Unheated	37	0 175	0 08
	5	0 095	
Heated	37	0 080	0 015
	5	0 065	

The media described in table 1 containing 0.1 per cent albumin, heated and unheated in 2 per cent NaCl, were stored for 20 days at 37 C and 5 C. Aliquots of 20 ml were then analyzed for free fatty acid.

The values obtained following storage at 5 C are only partly due to oleic acid, since albumin has a high blank value because of contamination by other ether soluble acids (Davis, 1947a). The slight hydrolysis obtained with heated albumin at 37 C compared with 5 C is probably spontaneous (Davis, 1947b), although the presence of a trace of residual lipase activity cannot be excluded. The increased titer obtained following incubation with unheated albumin at 37 C (0.08 ml greater than at 5 C) is due to hydrolysis of tween and corresponds to 0.002 per cent oleic acid in the medium, i.e., one-fifth of the tween present.

TABLE 6  
*Growth of minimal inocula*

ALBUMIN (0.1%)	INOCULUM (MG MOIST WEIGHT)			
	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>
	Growth*			
Unheated (fraction V)	4	3½	0	0
Unheated + 0.01% NaF	4	3½	3	0
Heated (56 C, neutral)	4	3½	3	0
Crystalline unheated	4	3	2	0

Human tubercle bacilli (H37Rv) were grown for 7 days in the tween-albumin medium, with the addition of a small amount of phlebotomist filtrate to promote dispersed growth (cf. section 7). The density of the growth in a portion of the culture was measured volumetrically after prolonged centrifugation to constant volume (1½ hours at 2,400 rpm) in a tube especially designed for this purpose, consisting of a capillary tube (0.9 mm in diameter) fused onto a wide (25 mm) test tube. The culture was adjusted to a concentration of 1 mm<sup>3</sup> of organisms per ml (i.e., 1 mg moist weight, or 0.2 mg dry weight, per ml) and serial 10-fold dilutions were made with especial care. Inocula were planted in tubes of media containing 0.05 per cent tween 80 and albumin and fluoride as indicated. Visible growth first appeared at 14 to 16 days, the results are recorded at 20 days.

\* Growth recorded at 20 days

containing a small number of tubercle bacilli counted under the microscope. The medium containing heated albumin developed visible growth regularly following inoculation with 2 or 3 bacilli with an incubation period approximately

the same as that of inocula of  $10^{-8}$  mg. The medium containing unheated albumin required larger inocula than did 2 or 3 bacilli (table 7). Technical limitations prevented testing of known inocula of 1 bacterial cell, which would undoubtedly have initiated growth as well as 2 cells in the stable lipase-free medium.

### *Effect of Horse Serum on Bacterial Growth*

The recognition of the lipase effect permitted an understanding of some hitherto puzzling observations, namely, the inhibitory effect of horse serum and of

TABLE 7  
*Inoculation of counted bacteria*

NUMBER OF BACTERIA INOCULATED	ALBUMIN (0.1%)	
	Heated	Unheated
20	+	+
10	+	+
4 (16/4)	+	+
4 (16/4)	+	—
3 (30/10)	+	—
3 (30/10)	+	—
3 (30/10)	+	—
3 (30/10)	+	—
2 (20/10)	+	—
2 (20/10)	+	—
2 (20/10)	+	—
2 (2/1)	+	—

A culture of human tubercle bacilli grown as in table 6 was lightly centrifuged to remove clumps of bacteria. The supernatant was diluted in water so that the number of bacteria in a tiny hanging drop could be counted microscopically under the high dry objective. Comparable drops of the diluent usually contained one or two particles which might have been confused with bacilli. Since it was not found possible to eliminate consistently such particles, it was not possible to isolate 1 or 2 bacilli accurately. Droplets containing larger numbers of bacilli were therefore transferred to given volumes of glucose solution, of which aliquots were then inoculated alternately into tubes containing medium with heated and unheated albumin. Growth became visible at 14 to 16 days and heavier at 20 days. Sterile water was added to replace that which had evaporated. Further observation until 25 days showed no growth in the tubes that were negative at 16 days.

+ = visible growth at 16 to 25 days

— = no visible growth at 25 days

certain bacterial culture filtrates on growth in the tween medium. Early in the course of studying possible growth factors for the tubercle bacillus, it was observed that the substitution of bovine or human serum for bovine serum albumin promoted growth, but of a flocculent rather than of a more or less dispersed type, horse serum, however, completely inhibited growth. The effect of horse serum was surprising since it has been used in the past in certain media designed for the cultivation of tubercle bacilli. It was even more surprising that, though horse serum is inhibitory in concentrations ranging from 2 to 10 per cent, a

higher concentration (20 or 40 per cent) permits growth of large inocula (table 8). The nature of the phenomenon was suspected following the further observations that sufficient bovine albumin overcomes the toxic effect and that in the absence of tween horse serum is not inhibitory (table 8).

TABLE 8  
*Bacteriostasis by horse serum*

SUBSTANCE ADDED TO 5 ML. MEDIUM	VOLUME ADDED (ML)	INOCULUM (MG MOIST WEIGHT)		
		10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
		Growth		
Medium containing 0.05% tween 80				
Control	0	3½	½	0
Albumin 5%	0.1	4	4	2
Bovine serum	0.1	4	0	0
	0.5	4	4	2
Human serum	0.3	4	4	3
Horse serum	0.1	0	0	0
	0.3	0	0	0
	1.0	4	0	0
	2.0	4	0	0
Heated horse serum (56 C, 30 min)	0.1	½	0	0
	0.3	4	0	0
	1.0	4	3	0
	2.0	2	1	½
Horse serum + 5% albumin	0.1 } 0.1 }	0	0	0
Horse serum + 5% albumin	0.1 } 0.5 }	4	3	3
Medium containing 0 tween 80				
Control	0	4	1	0
Horse serum	0.1	4	2	0

These results were explained when the horse serum used in these experiments was found to contain approximately three times as much lipase as did the human serum. The toxicity shown by horse serum but not by human serum can thus be accounted for by the higher concentration of lipase in horse serum, together with its known lower concentration of albumin (Moore, 1945). The disappearance of the toxicity of horse serum at very high concentrations occurs when the binding capacity of the albumin added in the serum exceeds the amount of tween

80 available as substrate for the lipase<sup>6</sup> The lipase in horse serum was apparently only partly destroyed by heating at 56 C for 30 minutes (table 8)

### *Bacteriostatic Effect of Bacterial Culture Filtrates*

Filtrates of the timothy grass bacillus (*Mycobacterium phlei*) have been reported to furnish an essential growth factor which permits *in vitro* cultivation of another mycobacterium, Johne's bacillus (Woolley and McCarter, 1940) The effect of such filtrates on tubercle bacilli was therefore studied *M phlei* was grown for 3 to 7 days in the tween-albumin medium, and the cultures were filtered through sintered glass (Corning UF) The sterile filtrates, however, not only did not promote growth of tubercle bacilli but were bacteriostatic in high dilutions Further studies revealed that the *phlei* filtrate was bacteriostatic only in the presence of tween 80 and that its bacteriostatic activity was destroyed by autoclaving It therefore appeared that we were dealing with lipase, rather than with a directly acting antibiotic agent This hypothesis was confirmed by chemical tests which showed that the *phlei* filtrate contained lipase in a much higher concentration than had been observed in any sera or in 5 per cent albumin

An extremely sensitive method of detecting lipase, in smaller concentrations than can be detected by any available chemical method, is offered by incubation of a tween medium (without albumin) with a bacterial filtrate (or another source of lipase), followed by inoculation with tubercle bacilli As little as 3 micrograms of oleic acid per ml, released following several days of incubation, can be detected The specificity of the bacteriostasis as a test for lipase can be tested by control experiments in a medium without tween By this means it was possible to demonstrate traces of lipase in the culture filtrate of an avian tubercle bacillus, but none in the filtrate of the human strain H37Rv

### *Dispersing Effect of Phlei Filtrate*

The growth of tubercle bacilli in the tween-albumin medium is only partly dispersed, most of the bacteria occurring in microscopic clumps (Middlebrook *et al*, 1947) It would be desirable for several purposes to secure completely dispersed growth The closest approach to this objective attained so far has been furnished by the addition to the medium of *phlei* filtrate in small amounts, which only slightly inhibit growth, the organisms grow in such a medium predominantly as single cells It has not been determined whether this dispersing effect is dependent upon the lipase in the *phlei* filtrate

The proper amount of *phlei* filtrate to provide dispersal without marked inhibition of growth must be empirically determined Excellent results have been obtained by adding to the medium, containing 0.05 per cent tween 80 and 0.2 per cent albumin, 5 to 10 per cent of its volume of the filtrate of a 3-day

<sup>6</sup> The lipolytic mechanism here suggested to account for the gross antibacterial action of horse serum, manifested only in the presence of tween 80, is not considered to conflict with numerous earlier reports that various sera, including antisera to tubercle bacilli, were inhibitory to tubercle bacilli in media without tween

culture of *M. phlei* grown in the same medium. The success of inocula consisting of very few cells from such a medium (tables 6 and 7) indicates clearly that though the phlei filtrate has a somewhat inhibitory effect on growth, the bacteria grown in this medium are perfectly viable.

#### DISCUSSION

It has been demonstrated that bovine serum albumin, although indispensable for the growth of small inocula of tubercle bacilli (especially in the "tween" medium), also exerts an inhibitory effect because of contamination of the commercial product (fraction V) by serum lipase, this enzyme releases free fatty acid in bacteriostatic concentrations from tween 80. After eliminating the lipase activity, growth can be initiated by smaller inocula (2 or 3 cells,  $10^{-3}$  mg moist weight) than were previously effective in the tween-albumin medium. A similar effect can be obtained in spite of the lipase by the use of very high concentrations of albumin, which are capable of binding all the fatty acid available from hydrolysis of the tween.

Since two bacilli initiate growth regularly, it is reasonably certain that single viable cells inoculated into the tween-albumin medium without lipase would also grow, the smallest successful inocula demonstrated here were limited to an average of two cells simply because of technical limitations. The reduction of the minimum effective inoculum from about 10 to only 1 or 2 bacteria is obviously of practical importance and will permit more effective use of the medium, or of solid modifications of it, in diagnostic or quantitative work.

The ability to initiate growth with very few cells also furnishes interesting information concerning the inoculum as well as the receptivity of the medium, namely, that no significant proportion of the cells of a 7-day culture in the tween-albumin medium is sterile. This behavior contrasts with the high proportion of sterile cells found in surface growth (Wilson and Schwabacher, 1936-1937).

Three methods have been found for eliminating lipase activity from albumin—the use of heated fraction V, of crystalline albumin, or of unheated fraction V with added NaF. In general, heated fraction V has seemed more useful than crystalline albumin. Not only is it less expensive, but it produces somewhat heavier growth of tubercle bacilli. The promotion of heavier growth has been traced to a dialyzable, thermostable contaminant of the fraction V that is absent from the crystalline albumin (Dubos, 1947). The third method of eliminating lipase activity, inhibition by addition of fluoride, has been shown to be effective in liquid media, but has not been extensively studied.

The deterioration of the tween medium on incubation with bacterial filtrates or other biological materials furnishes an exceedingly delicate test for lipase, in the absence of albumin, lipolysis, which yields as little as 3 micrograms of oleic acid per ml, can be detected by its inhibition of growth of tubercle bacilli. By this method no lipase could be detected in the filtrate of human tubercle bacilli. In view of the extraordinary sensitivity of this method, this observation suggests that the utilization of the oleic acid of tween 80 as a growth factor by the tubercle bacillus (Dubos, 1947) is not preceded by extracellular hydrolysis of the ester

in the medium The mechanism by which esterification permits fatty acids to be nutritive may thus be somewhat different from the effect of serum albumin, which reversibly binds fatty acid (Davis and Dubos, 1947) and presumably releases free fatty acid into the medium in extremely low concentrations as the fatty acid is utilized by the bacteria In the one case the organism would be expected to absorb the ester, in the other the free acid

It was observed that horse serum is bacteriostatic in a medium containing tween 80 but not in a medium without it The phenomenon can be accounted for by the relatively high concentration of lipase and low concentration of albumin in horse serum This observation is of general significance insofar as it emphasizes the need for guarding against lipolytic effects when a medium containing tween is inoculated with materials which may contain lipase (e g, in the cultivation of tubercle bacilli from blood or other tissues) In such circumstances it may be anticipated that the danger of bacteriostasis by free fatty acid can be avoided by the addition of fluoride or of sufficient albumin to bind all the fatty acid which may be released from the available tween Where dispersed growth is not desired, it has been shown that the oleic acid can be supplied as a growth factor by including oleic acid in the medium rather than tween 80, together with sufficient albumin to bind it, thus eliminating all danger of lipolysis (Dubos, 1947)

A bacteriostatic agent in filtrates of *Mycobacterium phlei* proved to be simply lipase, which released free fatty acid from tween 80 This phenomenon calls to mind the analogous result reported a few years ago for notatin (penicillin B), which proved to be glucose oxidase, its bacteriostatic effect arose from the formation of hydrogen peroxide in the presence of glucose (Birklinshaw and Raistrick, 1943) It may be anticipated that the tween-albumin medium will be used in chemotherapeutic testing, since it has several advantages for this purpose uniform inocula, uniform exposure of the bacteria to the medium, more rapid results, and ready quantitation of growth But in testing the activity of antibacterial agents of biological origin in this medium it is important, as shown by the experience with *phlei* filtrate, to distinguish lipolytic from direct antibacterial effects

#### SUMMARY

Commercial bovine serum albumin (fraction V), which permits growth of small inocula of tubercle bacilli in a liquid medium containing "tween 80" (a water-soluble ester of oleic acid), also makes the medium somewhat unstable This effect is due to the presence in the albumin of a small amount of lipase, which in 2 weeks releases enough fatty acid from tween to exceed the binding capacity of the albumin, the unbound oleic acid then reaches a bacteriostatic concentration

The lipase effect can be eliminated in several ways (1) destruction of the lipase by heating a neutral solution of fraction V at 56 C, which does not destroy the capacity of the albumin to bind fatty acid, (2) use of commercial crystalline bovine serum albumin, which is not perceptibly contaminated by lipase, (3)

addition to the medium of 0.01 per cent NaF, which inhibits lipase but is not bacteriostatic to the tubercle bacillus. The effect can also be overcome by the use of a high concentration of albumin.

When the lipase is eliminated by any of these methods, it is possible to initiate growth with smaller inocula ( $10^{-8}$  mg moist weight, or 2 cells) than are effective in the presence of unheated fraction V or in the absence of albumin.

Lipolytic activity also accounts for the bacteriostatic effect in this medium of horse serum and of a culture filtrate of *Mycobacterium phlei*. This experience emphasizes the importance of guarding against lipolytic effects when materials of biological origin are introduced into a tween-containing medium for diagnostic work (e.g., blood culture) or chemotherapeutic studies (e.g., impure antibiotics).

The use of "tween 80" and tubercle bacilli provides an exceedingly sensitive bioassay for lipase, which showed no trace of lipase in filtrates of cultures of growing human tubercle bacilli.

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# THE EFFECTS OF SALTS ON STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN AGAR PLATE ASSAYS<sup>1</sup>

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Experiments on the effects of salt on streptomycin in agar plate assays have produced conflicting observations. According to a recent paper by Berkman, Henry, and Housewright (1947), "the inclusion of 2.0 per cent sodium chloride, potassium chloride, or ammonium acetate and 0.85 per cent sodium chloride or ammonium acetate to the streptomycin sulfate solutions prepared for assay in 0.1 M phosphate buffer resulted in slightly decreased zones of inhibition." Conversely Loo *et al.* (1945) have stated that "addition of phosphate to streptomycin solutions caused a marked increase in the size of the zones of inhibition," and "an enhancing effect was also shown by sodium chloride, acetate, bicarbonate, and sulfate." Two such divergent conclusions on how salts in a solution of streptomycin influence the zones of inhibition made difficult any prediction of their behavior in agar plate assays. In this laboratory it has been found, however, that either result can be obtained from a streptomycin or dihydrostreptomycin solution containing salt; procedures are included in the following data.

In table 1 are given the average diameters of zones of inhibition, in mm, produced by a solution of dihydrostreptomycin<sup>2</sup> that contained 100 units per ml and 0, 0.8, or 1.6 per cent sodium chloride. The test agar contained 1.0 per cent peptone and 0, 0.8, or 1.6 per cent salt, and it was inoculated with the Waksman (1945) strain of *Escherichia coli*.

The zone diameters, in mm, given in table 2 were obtained by testing different concentrations of streptomycin in distilled water, 5 per cent sodium sulfate, or horse serum solution that had been diluted with an equal volume of distilled water adjusted to pH 7.4 with NaOH solution, or with a 0.1 M phosphate buffer at pH 7.4. The agar plates in which these solutions were tested were inoculated with spores of *Bacillus subtilis*.

The data show that the enhancing effect of salt depends on the salt concentration in the agar medium as well as on that in the solution assayed. An increasing salt concentration in the agar medium decreased inhibition zones produced by streptomycin and dihydrostreptomycin and lessened the enhancement of salts in the antibiotic solution. It has been tentatively concluded that the enhancement mechanism depends in part on the adsorbability of the medium.

When aqueous samples were diluted with phosphate buffer before being assayed, solutions which contained salt and 100 micrograms or more of streptomy-

<sup>1</sup> Part of this work, recommended by the Committee on Medical Research, was done under a contract between the Office of Scientific Research and Development and the University of California.

<sup>2</sup> Dihydrostreptomycin was obtained through the courtesy of Dr. Fred Stampert, Parke, Davis and Company, Detroit, Michigan.

cin per ml showed slightly decreased zones of inhibition. The decrease in diameter of the zone is negligible and has not been noticed in assays of solutions containing less than 50 micrograms of streptomycin per ml or in determinations made on agar media containing small amounts of salt. Although the smaller zones appear to be related to the influence of salts on the rate of diffusion and the rate of growth of the test organism, the exact causal mechanism is not clear.

In this laboratory, solutions of streptomycin, streptothricin, and dihydrostreptomycin are prepared for assay in agar plates according to the procedure of Loo *et al* (1945), but the tests are done in agar cups (Fleming, 1942), using Waksman's strain of *E. coli* in a 1 per cent peptone agar medium. The results are

TABLE 1  
*Effect of sodium chloride in the agar and in the solution assayed*

% NaCl IN AGAR	% NaCl IN SOLUTION		
	0.0	0.8	1.6
0.0	19.8 mm	21.5 mm	22.9 mm
0.8	16.5 mm	17.8 mm	17.9 mm
1.6	16.3 mm	16.7 mm	16.3 mm

TABLE 2  
*The effect of streptomycin concentration in water and phosphate*

Tested on salt-free agar medium						
CONCENTRATE STREPTOMYCIN MICROGRAM/ML	DILUTED WITH WATER			DILUTED WITH PHOSPHATE		
	Distilled Water	2.5% Na <sub>2</sub> SO <sub>4</sub>	Horse serum	Distilled water	2.5% Na <sub>2</sub> SO <sub>4</sub>	Horse serum
300	27.4 mm	31.0 mm	29.0 mm	28.3 mm	30.0 mm	27.8 mm
200	26.9 mm	29.6 mm	27.5 mm	26.6 mm	29.2 mm	26.6 mm
100	24.8 mm	26.7 mm	25.2 mm	25.0 mm	26.4 mm	24.6 mm
50	22.9 mm	24.4 mm	24.1 mm	23.6 mm	24.8 mm	24.3 mm
10	13.0 mm	14.5 mm	15.8 mm	17.0 mm	17.4 mm	17.2 mm
Tested on agar medium containing 0.8% sodium chloride						
200	23.2 mm	25.8 mm	25.3 mm	25.2 mm	25.9 mm	25.1 mm

satisfactory, except that even a tenfold difference in antibiotic concentration produces only a small difference in the diameter of the inhibitory zone.

A study of dihydrostreptomycin, including a more detailed account of the effects of salts, is in progress.

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# THE EFFECT OF AROMATIC DIAMIDINES ON BACTERIAL GROWTH

## I THE MECHANISM OF ACTION

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It was observed by Fuller (1942) that certain long-chained aliphatic diamidines exert an antibacterial action on gram-positive as well as on gram-negative microorganisms *in vitro*. Thrower and Valentine (1943) showed that propamidine (an aromatic diamidine, 1-1-diamidino-diphenoxy-propane), in addition to its high trypanocidal effect, also inhibits the multiplication of gram-positive cocci *in vitro* as well as *in vivo* in wounds and burns. These findings have been confirmed by many authors. The purpose of the experiments reported below was to test the influence of certain aromatic diamidines on bacteria *in vitro* and to study the mechanism of their action.

## EXPERIMENTAL RESULTS

Five aromatic compounds were tested: 4-4-diamidino-diphenyl-ether, 4-4-diamidino-diphenyl-benzyl-ether, 4-4-diamidino-diphenoxy-ether, 4-4-diamidino-stilbene (stilbamidine), and 4-4-diamidino-diphenoxy-pentane (pentamidine).

The microorganisms employed for the *in vitro* tests were *Escherichia coli* and *Staphylococcus aureus*. The medium used for the experiments with *E. coli* contained ammonium sodium phosphate, 0.5 per cent, sodium chloride, 0.2 per cent,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 per cent, and glucose, 0.2 per cent, whereas that employed for *S. aureus* was prepared according to Knight (1937). Both media were adjusted to pH 7.3 to 7.4. The experiments were conducted in standard test tubes and each contained 5.0 ml of medium to which phenol red had been added. The diamidines were added under sterile conditions. A quantity of each drug was weighed in a sterile Erlenmeyer flask, ether was then added to a depth of about 1 cm, and the flasks were kept in the dark until all the ether had evaporated. Sterile water was added to produce the required dilutions. Fresh solutions of drugs were prepared for each experiment.

*The influence of diamidine compounds on bacterial growth.* The bacterial suspensions were prepared as follows: bacteria were removed from a 20- to 24-hour agar slant with 10 ml of saline, and this stock suspension was diluted 1:10,000. One-tenth ml of this final dilution was added to each test tube. The results of the *in vitro* experiments were read after 24- and 48-hour incubation at 37°C. The minimal drug concentration at which no growth appeared after 48 hours was noted as the minimal inhibiting concentration. The results of these experiments are summarized in table 1.

Table 1 shows that the compounds most active against *E. coli* and *S. aureus* were stilbamidine and pentamidine. But when the test with *E. coli* was re-

TABLE 1  
Inhibition of bacterial growth by aromatic diamidines

ORGANISM TESTED	COMPOUNDS	MINIMAL EFFECTIVE MOLAR CONCENTRATION
<i>E. coli</i>	4-4-diamidino-diphenyl ether	$2 \times 10^{-4}$
	4-4-diamidino-diphenyl-benzyl-ether	$4 \times 10^{-4}$
	4-4-diamidino-diphenoxy-ether	$2 \times 10^{-3}$
	4-4-diamidino stilbene(stilbamidine)	$4 \times 10^{-3}$
	4-4-diamidino diphenoxy-pentane(pentamidine)	$4 \times 10^{-3}$
<i>S. aureus</i>	4-4 diamidino diphenyl-ether	$2 \times 10^{-4}$
	4-4 diamidino diphenyl-benzyl-ether	$2 \times 10^{-4}$
	4-4-diamidino-diphenoxy-ether	$1 \times 10^{-3}$
	4-4-diamidino-stilbene(stilbamidine)	$4 \times 10^{-3}$
	4-4-diamidino-diphenoxy-pentane(pentamidine)	$1 \times 10^{-3}$

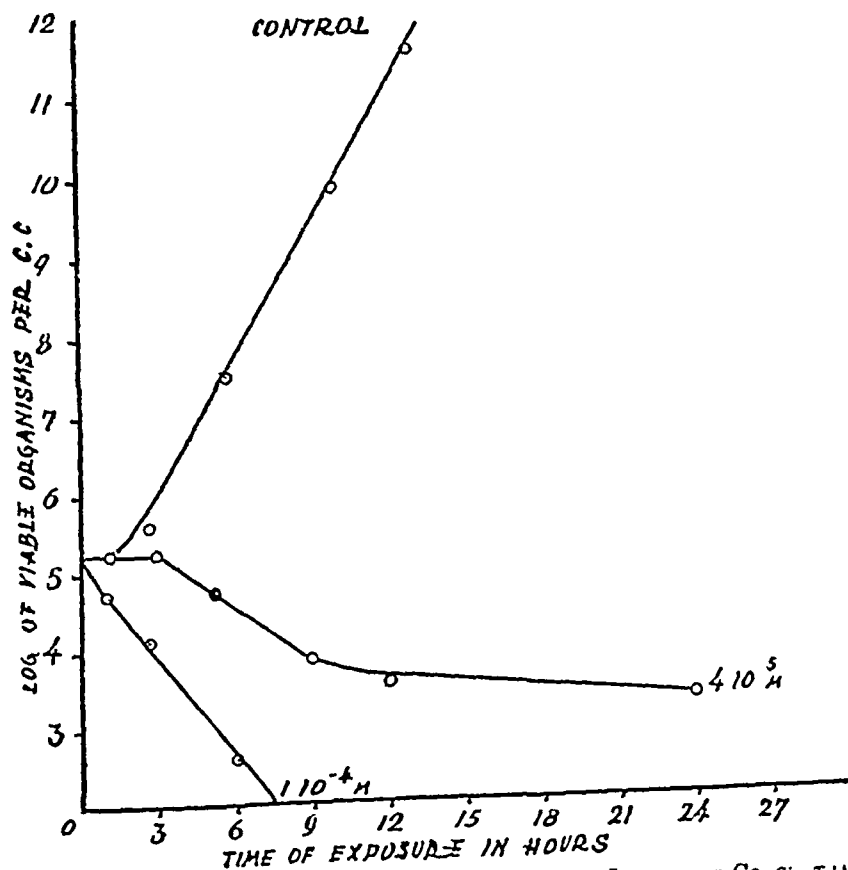


FIG 1 THE MAINTENANCE OF *E. COLI* IN THE PRESENCE OF INHIBITORY CONCENTRATIONS OF STILBAMIDINE

peated on Knight's medium (1937), the activity of these compounds was considerably depressed and their minimal effective molar concentration was  $1 \times 10^{-4}$

*Survival of microorganisms in the presence of diamidine compounds* In the following experiments only the most active compounds (stilbamidine and pentamidine) were employed. The purpose of these experiments was to determine whether the action of the compounds is bactericidal or bacteriostatic. Media and culturing procedure were identical with those in the experiment described

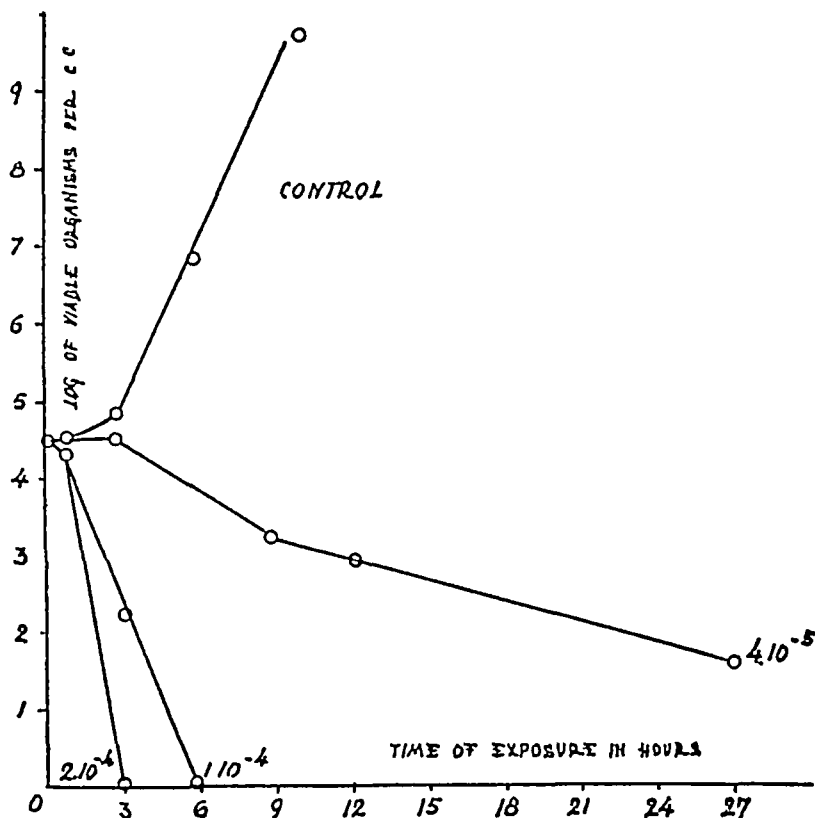


FIG 2 THE MAINTENANCE OF *E. COLI* IN THE PRESENCE OF INHIBITING CONCENTRATIONS OF PENTAMIDINE

above. After varying incubation periods at 37 C in the presence of varying quantities of drug, aliquot portions of the incubated mixtures were removed and the bacterial count was estimated by plating.

Figures 1 through 4 show the results of these experiments. There is full agreement between the actions of both compounds on *E. coli* suspended in its synthetic medium. Although the  $1 \times 10^{-4}$  molar concentration exerts a typical bactericidal effect, expressed by a logarithmic death curve, the  $4 \times 10^{-5}$  concentration, which we had found to be the minimal growth-inhibiting concentration, exerted quite a different effect. After an initial period lasting for about

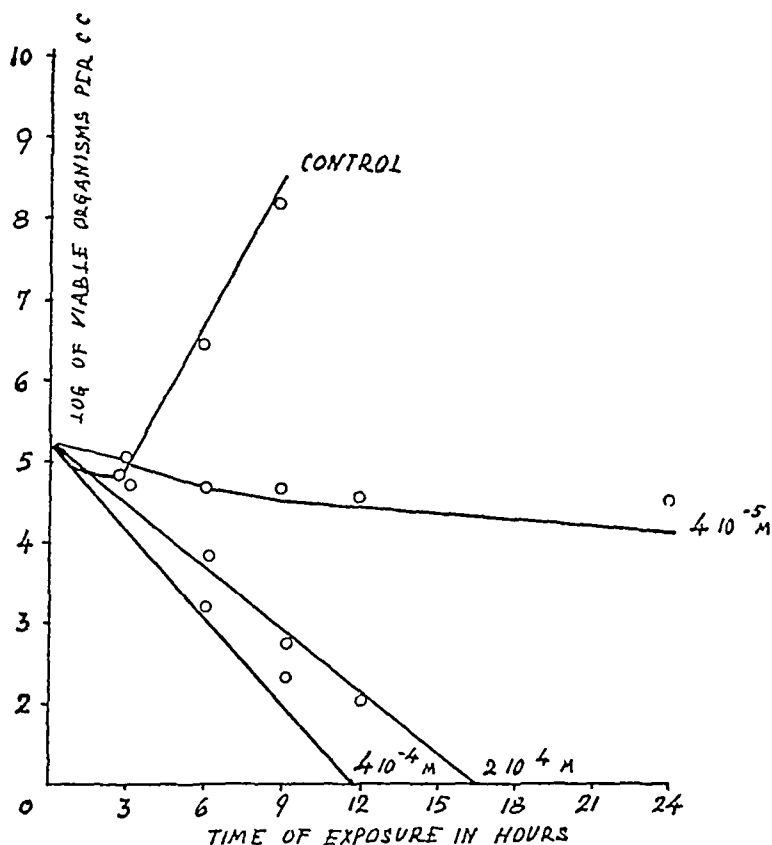


FIG 3 THE MAINTENANCE OF *S. AUREUS* IN THE PRESENCE OF INHIBITING CONCENTRATIONS OF STILBAMIDINE

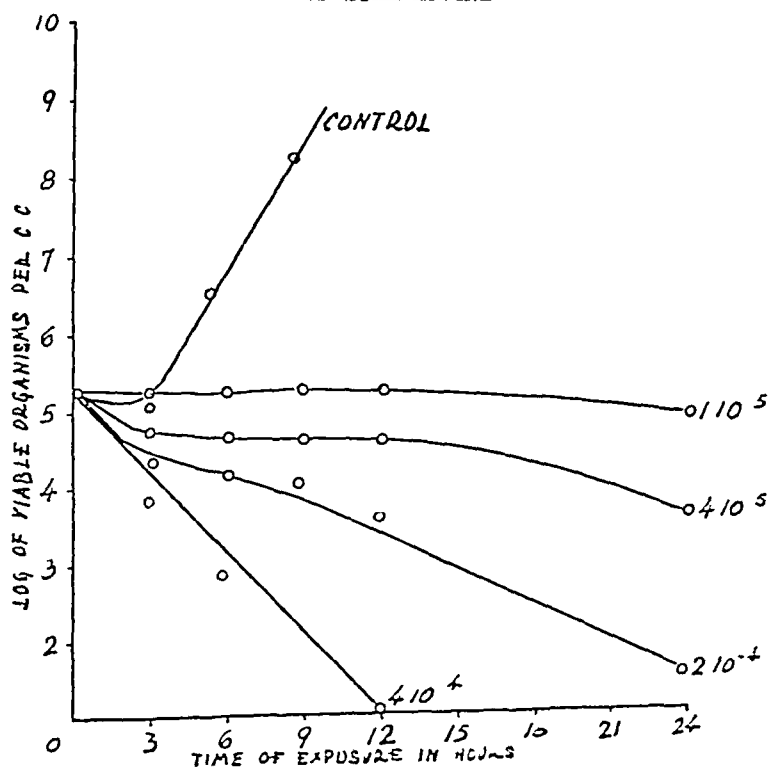


FIG 4 THE MAINTENANCE OF *S. AUREUS* IN THE PRESENCE OF INHIBITING CONCENTRATIONS OF PENTAMIDINE

3 hours, during which no change was noted in the bacterial count, there followed a period of about 6 hours during which the death rate was logarithmic, and this was followed by a period of low mortality, which lasted for at least 48 hours since at the end of this time living bacteria were still found. When these experiments were repeated on Knight's medium (1937), similar results were obtained, but higher drug concentrations were required to exert a bacteriostatic or a bactericidal effect.

*S. aureus* behaved quite differently in the presence of pentamidine and stilbamidine. The zone of drug concentrations inhibiting bacterial growth while permitting survival of the microorganisms after 48 hours was much wider than in the case of *E. coli*, as demonstrated in figures 3 and 4.

#### SUMMARY

Five aromatic diamidine compounds were tested for their antibacterial effect on *Escherichia coli* and *Staphylococcus aureus*.

Stilbamidine and pentamidine were found to be the most active of the five compounds.

Under identical conditions, *E. coli* is more resistant to the growth-inhibiting action of these compounds than is *S. aureus*. However, the zone of inhibition of growth unaccompanied by bactericidal action is narrower in the case of *E. coli* than in that of *S. aureus*.

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# THE EFFECT OF AROMATIC DIAMIDINES ON BACTERIAL GROWTH

## II THE ANTAGONISM OF NUCLEIC ACIDS AND POLYAMINES

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It is generally accepted that the composition of the medium exerts an important influence on the action of antibacterial substances on microorganisms. Many workers have succeeded in paralyzing the effect of antibacterials by the addition to the medium of substances such as organ extracts, peptone, and bacterial extracts. Thus, Mellman (1911) counteracted the antibacterial effect of acriflavin on *Escherichia coli* and on hemolytic streptococci by the addition of nucleotides and of a concentrate of amino acids. Lockwood (1938) inhibited the action of sulfonamides on hemolytic streptococci by the addition of serum and peptone. Stamp (1939) observed a similar effect after the addition of heated suspensions of hemolytic streptococci, and Woods (1910) after the addition of yeast extracts.

The assumption that the activity of these substances is associated with the presence of antagonists served as a starting point for the isolation and determination by Woods (1910) of *para*-aminobenzoic acid as the antagonist of sulfonamides.

In a preliminary paper (1911) we reported the antagonistic effect of peptone, meat extract, yeast extract, and particularly of nucleotides against the antibacterial activity of aromatic diamidines. Since then Snell (1944) has reported on the antagonistic effect of some polyamines against the antibacterial action of propamidine on lactobacilli. The mechanism of the action of nucleotides against the antibacterial action of diamidines is the subject of the present study.

### EXPERIMENTAL

In our *in vitro* experiments with *E. coli* and *Staphylococcus aureus* we followed the technique described by us in a previous communication. The present experiments were carried out with the drugs formerly found to be the most active, stilbamidine and pentamidine. With the exception of blood and serum the antagonists were autoclaved at 15 pounds' pressure for 15 minutes. In order to ensure contact between the microorganisms and the antibacterials the former were inoculated into test tubes containing the medium with varying quantities of diamidines, and only after 15 minutes' contact between bacteria and antibacterials at 37 C was the antagonist added. The results were recorded after 24 as well as after 48 hours.

*Antagonistic action of chemically undefined substances against antibacterials*  
Experiments with substances of this group are summarized in table 1, which shows that peptone, bovril, marmite, and acid casein hydrolyzate inhibit the

antibacterial effect of diamidines to a remarkable degree. The effect of pentamidine on *S. aureus* was not markedly reduced by any of the antagonists, but the inhibition of the effect of the same drug on *E. coli* was well marked. The effect of stilbamidine on both microorganisms was antagonized to nearly equal degrees by the different substances.

*Antagonistic action of substances of similar structure* In view of the fact that the antibacterials contained the amidine group, we attempted to learn whether other compounds which contain a similar structure are able to inhibit antibacterials. Guanidine, arginine, creatine, and creatinine were tested and found to be inactive.

*Antagonistic action of amino acids* The following amino acid concentrations were also found to be inactive: glycine, 1.0 per cent, *dl*-alanine, 0.5 per cent,

TABLE 1

*Influence of a series of chemically undefined substances on the antibacterial action of diamidines*

ANTAGONISTS	CONCENTRATION OF ANTAGONISTS	MINIMAL MOLAR CONCENTRATION INHIBITING GROWTH OF			
		<i>S. aureus</i>		<i>E. coli</i>	
		Pentamidine	Stilbamidine	Pentamidine	Stilbamidine
	<i>per cent</i>				
Control		$1 \times 10^{-5}$	$4 \times 10^{-5}$	$4 \times 10^{-5}$	$4 \times 10^{-5}$
Difco peptone	5.0	$1 \times 10^{-5}$	$4 \times 10^{-5}$	$2 \times 10^{-4}$	$4 \times 10^{-4}$
	1.0	$1 \times 10^{-5}$	$2 \times 10^{-4}$	$1 \times 10^{-4}$	$1 \times 10^{-4}$
Bovril	2.0	$2 \times 10^{-5}$	$1 \times 10^{-3}$	$1 \times 10^{-4}$	$1 \times 10^{-3}$
	1.0	$1 \times 10^{-5}$	$2 \times 10^{-4}$	$4 \times 10^{-5}$	$2 \times 10^{-4}$
Marmite	2.0	$2 \times 10^{-5}$	$1 \times 10^{-3}$	$2 \times 10^{-4}$	$1 \times 10^{-3}$
	1.0	$1 \times 10^{-5}$	$2 \times 10^{-4}$	$1 \times 10^{-4}$	$4 \times 10^{-4}$
Acid casein hydrolyzate	3.0	$1 \times 10^{-5}$	$1 \times 10^{-4}$	$4 \times 10^{-4}$	$1 \times 10^{-3}$
Rabbit serum	10.0	$1 \times 10^{-5}$	$4 \times 10^{-5}$	$4 \times 10^{-5}$	$6.6 \times 10^{-5}$
Defibrinated rabbit's blood	10.0	$1 \times 10^{-5}$	$4 \times 10^{-5}$	$6.6 \times 10^{-5}$	$6.6 \times 10^{-5}$

*dl*-methionine, 0.35 per cent, *l*-tryptophane, 0.1 per cent, *dl*-phenylalanine, 0.15 per cent, *dl*-2-amino-*n*-valeric acid, 0.1 per cent, *l*-oxyproline, 1.0 per cent, *l*-proline, 0.1 per cent, histidine hydrochloride, 0.01 per cent, sodium glutamate, 0.2 per cent, cystine hydrochloride, 0.01 per cent, and lysine dihydrochloride, 0.1 per cent.

*Antagonistic action of nucleic acid* Hawking and Smiles (1941) studying the distribution of stilbamidine in trypanosomes found that this fluorescent substance tends to concentrate in the blepharoplast and in certain plasma granules. Adler and Tchernomoretz (1940) noted that, after treatment of certain babesia infections with stilbamidine, the first signs of degeneration occur in the nuclei of the parasites. This observation led to the assumption that the drug may be concentrated in a structure rich in nucleic acids.

These experiments together with those reported above on the antagonistic action of substances like marmite and bovril, which are rich in nucleic acids, led

us to examine the antagonistic effect of nucleotides Sodium nucleate was employed in the following experiments

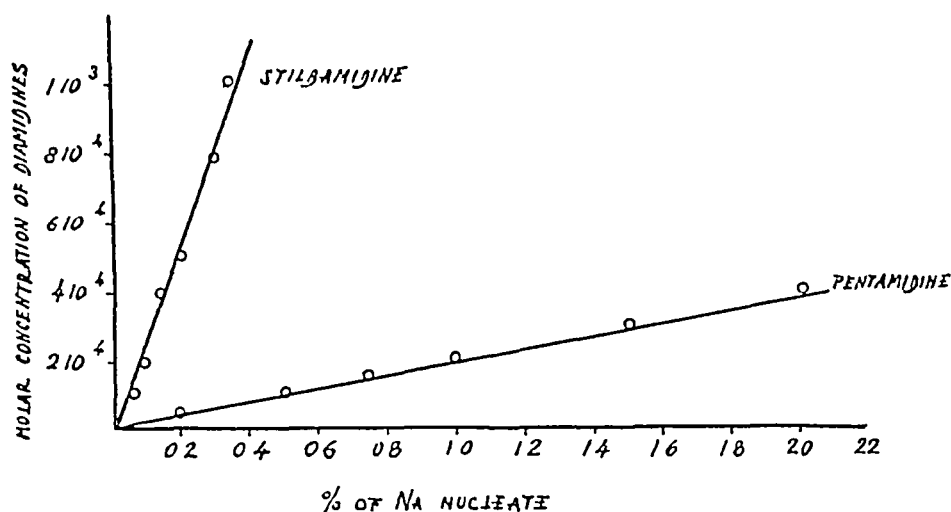


FIG 1 ANTAGONISTIC ACTION OF SODIUM NUCLEATE AGAINST AROMATIC DIAMIDINES IN *E. COLI*

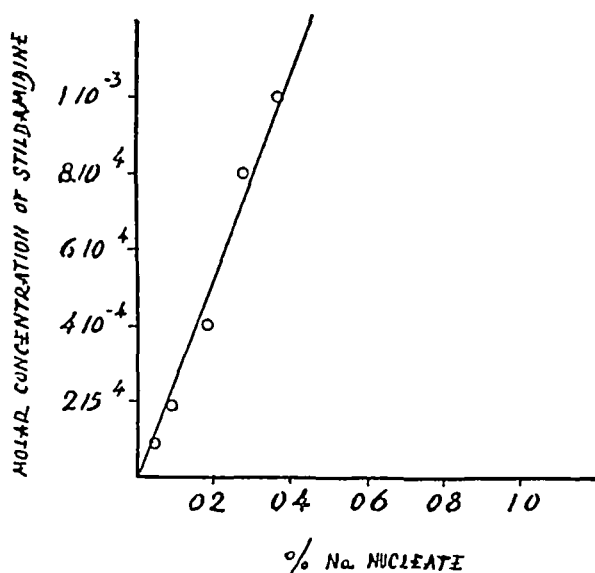


FIG 2 ANTAGONISTIC ACTION OF SODIUM NUCLEATE AGAINST STILBAMIDINE ON *S. AUREUS*

As demonstrated in figures 1 and 2 sodium nucleate exerts an antagonistic action against the antibacterial activities of stilbamidine and pentamidine on the growth of *E. coli*. This effect is demonstrable through a wide range of antibacterial drug concentrations if a suitable concentration of the antagonist is

employed But the concentrations required to inhibit the effect of pentamidine are higher than those required for the inhibition of the stilbamidine

When employed against *S. aureus*, stilbamidine is inhibited by sodium nucleate, whereas pentamidine is so inhibited only in its lowest effective concentrations, and even in this zone high concentrations of sodium nucleate are required Nucleic acids of the desoxyribonucleic type behaved similarly

*Antagonistic action of accessory growth substances* In order to exclude the possible effect of the contamination of nucleotides of natural origin with minimal quantities of accessory growth substances, we examined the antagonistic effects of a series of well-known growth promoters in molar concentrations ranging from  $1 \times 10^{-4}$  to  $1 \times 10^{-2}$  The following substances were tried and found to be inactive as antagonists of diamidines: thiamine, riboflavin, niacin, choline, pimelic acid, calcium pantothenate, biotin, pyridoxine, para-aminobenzoic acid, and inositol

*Antagonistic action of the constituents of nucleic acid* In order to determine whether the antidiamidine effect is ascribable to the nucleic acid molecule as a whole or to its component parts, we examined the antagonistic action of the constituents of the polynucleotide Of the purine and pyrimidine bases we examined adenine, guanine, xanthine, hypoxanthine, and uracil These substances, which are soluble with difficulty in water, were examined in varying dilutions of saturated solutions All of them proved to be inactive

Of the nucleosides we examined adenosine and guanosine in concentrations up to 5 per cent Both substances proved to be inactive On the other hand, the mononucleotides, adenylic and guanylic acid, showed a certain activity (figure 3), which was, however, far from that of the polynucleotide, the active concentration of which by weight was 15 to 20 times lower Mixtures of the two mononucleotides were no more effective than was each mononucleotide taken separately

These different degrees of activity are evidenced not only in differences of active concentrations but also in velocities of action In the case of the inhibition of diamidines by polynucleotides bacterial growth appeared as early as 24 hours after inoculation, whereas in the case of mononucleotides growth in the corresponding diamidine dilutions occurred only after 2 to 3 days

*Antagonistic effect of an alkaline hydrolyzate of nucleic acid* Since we had only two mononucleotides at our disposal, we determined whether the antidiamidine effect is associated with the undestroyed polynucleotide molecule as a whole or with its constituents obtained by hydrolysis with ammonium hydroxide Five g of nucleic acid were dissolved in 26.5 ml of 25 per cent ammonium hydroxide, and this mixture was autoclaved at 15 pounds for 30 minutes No difference in activity was observed between the original polynucleotides and the product obtained after hydrolysis

*Antagonistic effect after the delayed addition of nucleic acid* When higher concentrations of diamidines, especially stilbamidine, come in contact with nucleic acids a precipitate appears Since nucleosides do not give this reaction, whereas, on the other hand, mononucleotides and salts of phosphoric acid do,

it seemed likely that the phosphorus group of the nucleotides was responsible for the complexes formed

The question therefore arose whether the antidiadimine activity of nucleotides was associated with the formation of these insoluble complexes, formed before the antibacterial substance had the opportunity to combine with the constituents of the bacterial body. We brought the bacteria into contact with the inhibiting concentrations of diadimine for 24 hours at 37 C and subsequently added the concentrations of nucleic acids known to be antagonistic. After an additional incubation of 24 hours, bacterial growth appeared in the same zone as that

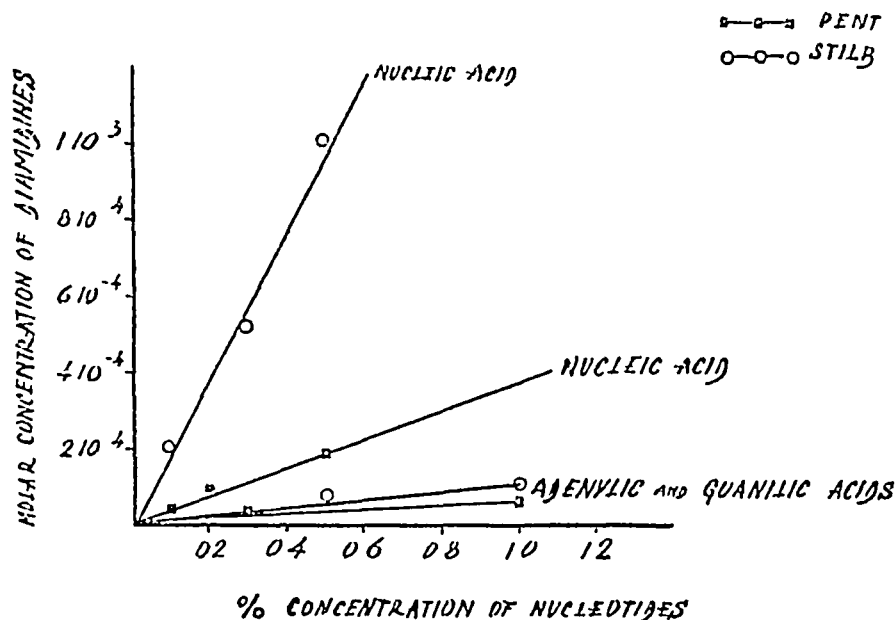


FIG 3 ANTIDIADIMINE ACTIVITY OF THE POLYNUCLEOTIDES AND MONONUCLEOTIDES ON *E. COLI*

observed if the nucleic acid was added immediately after the antibacterial substance

*Antagonistic effect of polyamines* While this work was in progress the reports of Silvermann and Evans (1944) and of Snell (1944) appeared on the antagonistic activity of certain polyamines against the antibacterial action of atabrine, quinine, and propamidine. Comparative experiments were therefore undertaken to determine whether differences exist in the mode of action of these different groups of antagonists.

Spermine and spermidine phosphate (Hoffmann-La Roche) and the synthetic polyamines, diethylene triamine (DT), triethylene tetra-amine (TT), and tetra-ethylene penta-amine (TP) from the Carbon and Carbide Chemical Company, were employed. It was found that spermine and spermidine, in concentrations of  $5 \times 10^{-4}$  molar, inhibit bacterial growth, and that in lower concentrations

they exert no antagonistic effect. On the other hand, the synthetic products (DT, TT, and TP) had no antibacterial effect even if employed in a  $1 \times 10^{-1}$  molar concentration, and in molar concentrations between  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$  they had an antagonistic effect.

There is a certain correlation between antagonistic activity and length of the hydrocarbon chain of the polyamine. The most active compound was TT. Comparing the effects of the synthetic polyamine and of the nucleic acids, we

TABLE 2  
*Antagonistic action of polyamines against the inhibition of growth by diamidine*

POLYAMINES	MOLAR CONCENTRATION OF POLYAMINES	MINIMAL MOLAR CONCENTRATION INHIBITING			
		<i>E. coli</i>		<i>S. aureus</i>	
		Stilbamidine	Pentamidine	Stilbamidine	Pentamidine
Diethylenetriamine	$1 \times 10^{-1}$	$1 \times 10^{-4}$	$2 \times 10^{-4}$	$8 \times 10^{-5}$	$2 \times 10^{-5}$
	$5 \times 10^{-1}$	$8 \times 10^{-4}$	$1 \times 10^{-4}$	$4 \times 10^{-5}$	$1 \times 10^{-5}$
	$1 \times 10^{-2}$	$4 \times 10^{-5}$	$8 \times 10^{-5}$	$4 \times 10^{-5}$	$1 \times 10^{-5}$
Triethylenetetramidine	$1 \times 10^{-1}$	$2 \times 10^{-4}$	$5 \times 10^{-4}$		
	$5 \times 10^{-1}$	$2 \times 10^{-4}$	$2 \times 10^{-4}$	$2 \times 10^{-4}$	$4 \times 10^{-5}$
	$1 \times 10^{-2}$	$1 \times 10^{-4}$	$1 \times 10^{-4}$	$1 \times 10^{-4}$	$2 \times 10^{-5}$
Tetraethylenepentamidine	$1 \times 10^{-1}$	$2 \times 10^{-4}$	$5 \times 10^{-4}$		
	$5 \times 10^{-1}$	$2 \times 10^{-4}$	$2 \times 10^{-4}$	$2 \times 10^{-4}$	$4 \times 10^{-5}$
	$1 \times 10^{-2}$	$1 \times 10^{-4}$	$1 \times 10^{-4}$	$1 \times 10^{-4}$	$2 \times 10^{-5}$
Control		$4 \times 10^{-5}$	$4 \times 10^{-5}$	$4 \times 10^{-5}$	$1 \times 10^{-5}$

TABLE 3  
*Antagonistic action of nucleic acid against the antibacterial action of natural polyamines*

CONCENTRATION OF NUCLEIC ACID	GROWTH OBSERVED IN A MAXIMAL MOLAR CONCENTRATION OF	
	Spermidine	Spermine
<i>per cent</i>		
0.1	$5 \times 10^{-5}$	$5 \times 10^{-5}$
0.05	$5 \times 10^{-4}$	$1 \times 10^{-5}$
0.01	$1 \times 10^{-4}$	$5 \times 10^{-4}$
Control	$1 \times 10^{-4}$	$2 \times 10^{-4}$

find that the former act only in the narrow zone of the lowest inhibiting diamidine concentration, whereas the latter also act in multiple antibacterial concentrations.

*Antagonism of nucleic acid against the antibacterial action of spermine and spermidine on E. coli.* In the following experiment we tried to inhibit the antibacterial action of spermine and spermidine with nucleic acids. The results are summarized in table 3. Table 3 shows that a 0.05 per cent concentration of nucleic acid is sufficient to paralyze the effect of either of the natural polyamines, spermine and spermidine.

## DISCUSSION

Our observations on the antagonistic effect of nucleic acids against the antibacterial action of diamidines resemble, in many respects, those of Fildes (1940) on mercuric compounds. In both cases antibacterial activity was inhibited by direct chemical inactivation. It seems that a direct chemical reaction between nucleic acids and the diamidine led to the formation of insoluble complex compounds. It may be that diamidines that have passed through the cell wall also react primarily with cellular constituents composed chiefly of nucleotides, thereby interfering with the vital functions of these cell constituents. The addition of such metabolites to the medium should enable the cell to restore its normal functions. The assumption that extracellular inactivation of diamidine occurred before it had succeeded in entering the cell was at variance with the experimental results, since even after 24-hour contact between the bacterial cell and the antibacterial drug it was possible to inhibit the action of the antibacterial by the addition of nucleic acids. This fact does not preclude the possibility that even after contact between the receptive constituents of the bacterial cell and the antibacterial the presence of an anionic compound in excess may change the chemical or adsorptive equilibrium between the bacterial cell and the antibacterial.

The main question is whether this antagonism between the diamidine and the nucleic acid is a specific one or whether the phenomenon under discussion is the result of a nonspecific ionic exchange between two competing substances. A nonspecific competitive ion exchange would be indicated if the action of a toxic cationic compound, which exerts an affinity for some anionic constituents of the cell, is inhibited by the addition of nontoxic anion competing with these critical points in the cell for the toxic substance without fulfilling any function in the cell metabolism. The mononucleotides (adenylic and guanylic acids) also form insoluble complexes with diamidines *in vitro* in suitable concentrations. However, their antagonizing activities are not comparable with those of the polynucleotides, since more than 20 times higher concentrations of the mononucleotides are required to yield an effect similar to that of the polynucleotides, and the activity of the former is expressed by a delayed bacterial growth which appears only after 2 to 3 days.

These facts lead us to assume that the role played by the polynucleotides is specific and is, furthermore, not bound to the intact molecule but to the sum of its constituents, since equal effects were obtained with intact or with hydrolyzed products. It seems that the nucleic acids or their constituents play a specific role in bacterial metabolism and that their presence helps the bacterial cell to overcome the damage caused by the antibacterials.

The concentrations required for this protective effect, although they are rather high, are of the same order of magnitude as those of nucleic acids in the bacterial cell. Thus, Thompson and Dubos (1938) found 2 to 5 per cent of nucleic acid in the dried substance of pneumococci, and Johnson and Coghill (1925) found 0.42 per cent in *Mycobacterium tuberculosis*. The antagonism of nucleic acid against the antibacterial action of the diamidines is exerted through a long range

of concentrations of both these substances, and the ratio of the concentration of nucleic acid required to inhibit a given concentration of the antibacterial remains constant. Only in the case of pentamidine activity on *S. aureus* did we succeed in abolishing its action in a narrow zone of the lowest inhibiting concentrations ( $1 \times 10^{-5}$  to  $4 \times 10^{-5}$ ), and this was accomplished only with relatively high concentrations of nucleic acid (1 to 2 per cent). Pentamidine may possess a strong affinity for the nucleotide centers of the cell of *S. aureus*, and large amounts of nucleic acid may therefore be needed to overcome this affinity. But it is not excluded that centers other than nucleotides may also be attacked by pentamidine and that this additional action may not be antagonized by nucleic acids.

The observation of Kopac (1946) on the dissociation of protamine nucleate complexes by stilbamidine supports this assumption and may serve as a new starting point for further investigation on the mode of action of diamidine on nuclear structures. The observation of Hawking and Smiles (1941) on the affinity of stilbamidine for the blepharoplasts of trypanosomes, and for certain granular constituents of the plasma, and the observation by Adler and Tchernomoretz (1940) that diamidines primarily attack the nuclei of babesia further support our assumption.

We are led to assume that quite a different mechanism of action exists in the antagonism between diamidines and polyamines. In this case both substances are cationic in nature and are somewhat similar in structure. The existence of a competitive cationic exchange, as proposed by Vallo and Du Bois (1941), seems to provide us with an adequate explanation. The problem is whether the phenomenon under discussion is a specific one and whether the polyamines or certain substances of similar chemical structure play a specific role in bacterial metabolism, as assumed by Silvermann and Evans (1944), or whether the phenomenon is nonspecific (Elson, 1945) and caused by relatively atoxic cationic compounds in a manner similar to the antagonistic effect exerted by certain atoxic cationic compounds on cationic detergents.

The fact that nucleic acid antagonizes the inhibiting effect of diamidines as well as polyamines (spermine and spermidine) on the growth of *E. coli* may serve as a clue to the fact that both inhibiting substances have a specific affinity for the same cell constituents, whether or not the polyamines play any role in bacterial metabolism.

#### ACKNOWLEDGMENT

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#### SUMMARY

Many complex substances such as marmite, peptone, and beef extract parallelize the growth-inhibiting effect of diamidines on *Escherichia coli* and *Staphylococcus aureus*. Neither blood nor serum in concentrations up to 10 per cent neutralize such an effect.

Among a series of essential metabolites only, the nucleic acids derived from



yeasts or animal tissues were able to inhibit the antibacterial effect of diamidines Guanidine, arginine, creatine, creatinine, and other amino acids, and the vitamins of the B group exerted no such effect

The antidiamidine effect of the nucleic acids was observed through a wide range of concentrations of the antibacterial substances Hydrolyzed nucleic acids exerted the same effect as the untreated products The activity of the mononucleotides (adenylic and guanylic acid) was about one-twentieth that of the polynucleotides Among the nucleosides guanosine and adenosine were examined These substances, as well as the purine bases, were devoid of any activity

The natural polyamines (spermine and spermidine) in high concentrations themselves exerted an antibacterial effect, and in low concentrations they had an antidiamidine effect The synthetic products, diethylenetriamine, triethylenetetra-amine, and tetraethylenepentamine, exerted an antidiamidine effect against the highest dilutions of the antibacterial substances

The mechanism of the antidiamidine activity is discussed It is assumed that the diamidines cause metabolic disturbances of the cell nucleotides by fixation of nuclear substances The antagonism of the polyamines seems to be associated with their competition for the same cellular substance

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# A NOTE ON DETERGENT INTERFERENCE IN THE SERIAL DILUTION ASSAY OF PENICILLIN USING *BACILLUS SUBTILIS*

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The merit of the serial dilution method employing *Bacillus subtilis* for the assay of penicillin in blood as originally described by Randall, Price, and Welch (1945) has been a subject on which opinions differ. It has recently been claimed by Buggs *et al* (1946), Chandler *et al* (1945), and Elias *et al* (1945) that a fairly extensive normal blood bacteriostasis sometimes exists against *B. subtilis*. This alleged inhibition has been disputed by Hoffman and Volini (1947) and by Dolkart *et al* (1947), who found the method reliable for human blood assays. Subsequently, Hoffman *et al* (1947) found in 100 plasma samples relatively high in penicillin that, after adequate penicillinase inactivation, the assay was always zero. The method had also been tested against 50 normal rabbit plasma samples in our laboratories. No significant bacteriostasis was found in any of these samples against *B. subtilis*. Furthermore, in our routine rabbit blood analyses, positive controls have been rare. Only once was an extraneous bacteriostasis encountered, and this, as is shown in this paper, was caused by the glassware cleaning agent.

The blood assay method employed in our laboratories has generally been a mechanized adaptation of the method of Randall, Price, and Welch (1945). *B. subtilis* NRRL-B-558 was the test organism. A Brewer automatic pipetting machine was employed for dispensing all constant volumes required. Wire racks holding 2 to 4 series of ten 12-by-100-mm test tubes or Kahn tubes were used. The racks of tubes were covered with rectangular, noncorroding metal covers, preferably stainless steel. These fit the racks well enough that plugging of the tubes was unnecessary to maintain sterility for the period of assay. The absence of plugs was essential for efficient mechanical operation. The method was described briefly by Hoffman and Volini (1947).

During the period when the method was being established at the Hektoen Institute, Cook County Hospital, Chicago, some very disturbing irregularities were observed in the growth of *B. subtilis* in the serial dilution tubes. Tubes showing growth were found intermingled at random with clear tubes showing no growth. This occurred even when inoculated broth alone was dispensed into penicillin-free assay tubes. At 2 ml of broth per tube, growth was erratic. At 4 ml of broth per tube, growth occurred in nearly all tubes. At about 0.5 to 1.0 ml per tube there was no growth in practically all tubes. Suspicion of the source of interference of growth was cast, therefore, upon the glassware, and upon its cleaning agent in particular.

As a cleaning agent, "calgonite" had been employed. This material con-

tained alkaline silicates, alkaline hexametaphosphates, and other agents. Upon cleaning, rinsing up to six times, and drying, the glassware was sparkling. This was somewhat misleading because it was found that some of the cleaning agent must have remained even after the normally extensive rinsing. It presumably entered the assay medium and in some manner suppressed the growth of *B subtilis* irregularly, depending apparently upon the amount of agent released into the solution. The addition of blood plasma or serum counteracted this inhibitory effect to a degree depending upon its concentration.

The use of other detergents such as the sodium alkyl sulfate type (e.g., "duponol") in low concentration was generally satisfactory. Tubes with a visible hard-water film were usually safer to use than some of the sparkling tubes with an invisible, glassy, adherent film. The fact that films of extraneous materials on glassware can exert a deleterious effect on the growth of the tubercle bacillus has been pointed out by Dubos and Davis (1946).

Our experience has shown that it is possible that a residual cleaning agent adhering to the glassware may render the *B subtilis* serial dilution assay for penicillin of doubtful value. For satisfactory results a detergent of the sodium alkyl sulfate type is recommended.

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# THE USE OF THE ELECTRON MICROSCOPE IN DIAGNOSIS OF VARIOLA, VACCINIA, AND VARICELLA

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The clinical differential diagnosis of variola, or generalized vaccinia, and varicella in their early stages is often extremely difficult, and clinically mild cases of variola may be indistinguishable from varicella throughout the course of the disease. Laboratory methods of diagnosis such as flocculation, complement fixation tests, and Paul's rabbit cornea test are only applicable for the diagnosis of variola in the later stages of the disease.

Paschen (1906) described elementary bodies of vaccinia in smear preparations of vesicle fluids for microscopy and later (1919) observed what he believed to be elementary bodies of varicella, which he found to be much smaller in size and of a lower staining affinity than those of vaccinia. Amies (1933) and van Rooyen (1944) confirmed Paschen's findings as to the existence of elementary bodies of varicella in vesicle fluids. Van Rooyen utilized both the difference in the morphology and the frequency of occurrence of the elementary bodies of variola and varicella as a clinical diagnostic method for the differentiation of these diseases in man.

Since the introduction of the electron microscope, this instrument has been employed extensively in morphological studies of viruses, but no studies have yet been published on its application as a diagnostic tool in the differentiation of virus diseases, such as variola and varicella.

During a recent outbreak of smallpox in New York we had the opportunity to collect specimens for electron microscopy of human tissue from clinical cases of variola, generalized vaccinia, and also varicella, and in this paper will be presented electron micrographs derived from these specimens. For comparison are also presented electron micrographs of the virus of vaccinia as prepared from epidermal tissue of a calf and as found in stock calf lymph vaccine for human vaccination against smallpox. The studies were conducted with a RCA electron microscope, type EMU, and the shadow-casting technique of Williams and Wyckoff (1945) has been employed in the production of the metal-shadowed micrographs.

## MATERIALS AND METHODS

A specimen presumed to contain elementary bodies of variola was obtained through the courtesy of Drs. Ralph Muckenfuss and George Hirst of the Willard Parker Hospital, New York. This material was recovered from a nonfatal human case on the twentieth day of the disease.

Crusts were collected, ground with silicate, suspended in saline, and centrifuged

at 2,500 rpm for 20 minutes. The elementary bodies were purified by a second centrifugation at 15,000 rpm for 1 hour. The sediment was finally resuspended in 0.9 ml distilled water, thus concentrating the suspension to one-tenth of its original volume. A small drop of the final suspension was then placed on the collodion-covered screens and dried in the usual manner.

We are greatly indebted to Drs. Emmett Holt, Jr., and Robert Ward of the Bellevue Hospital, New York, for two samples from a 10-year-old child who showed multiple open craterform pustules on the vulva 12 days after she had been vaccinated against smallpox. One sample was taken with a cotton swab from the site of the vaccination after the well-formed scab had been removed. The second sample was obtained, also with a swab, from the pustular eruptions in the vulval region. These swabs were washed in solutions of physiologic saline and the resulting suspensions were purified by differential centrifugation.

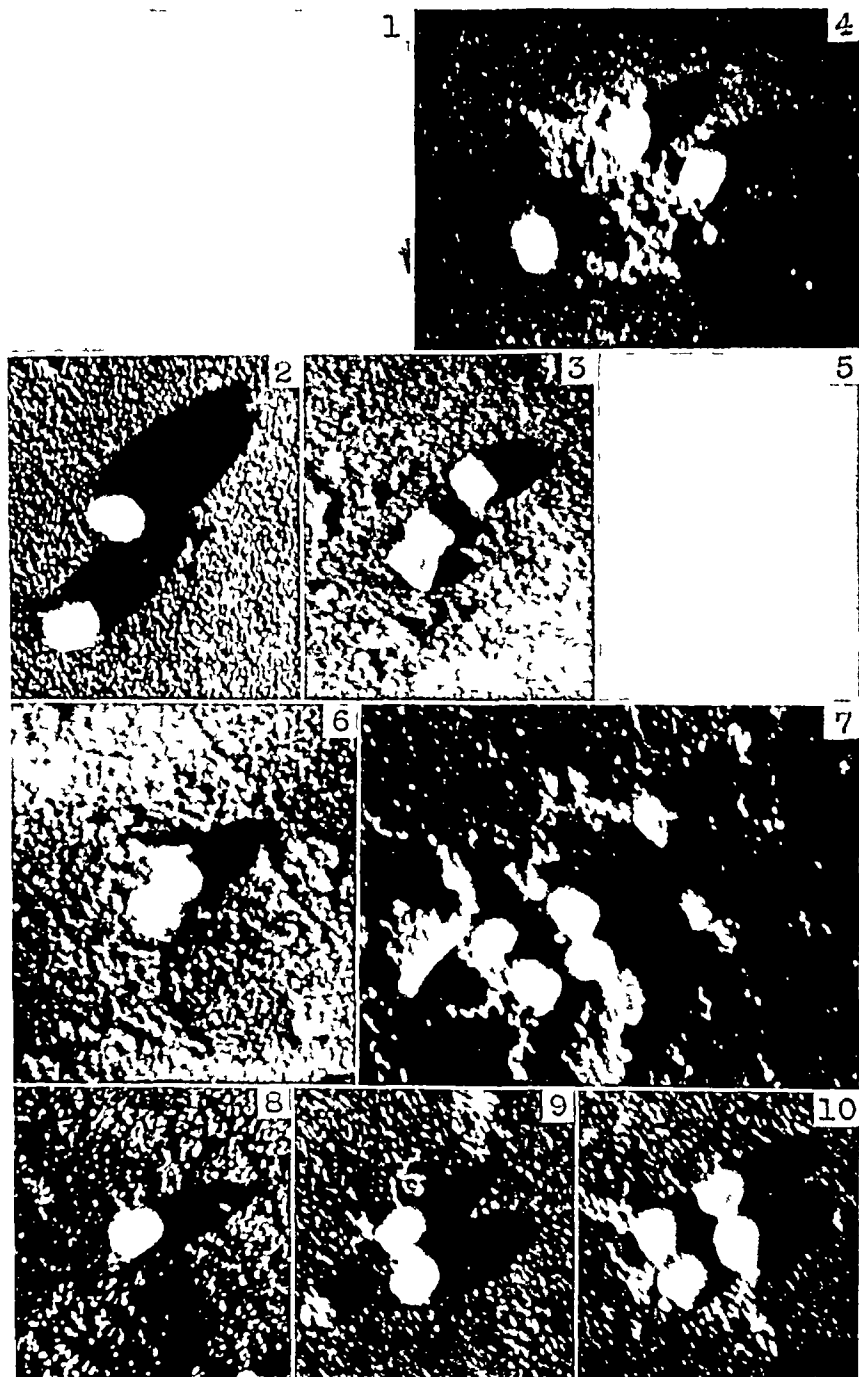
A suspension of vaccinia virus for electron microscopy was kindly provided by Dr. W. Koch of the Biological Division, E. R. Squibb and Sons, New Brunswick. It was recovered from a calf which had been infected intradermally with this agent for routine vaccine production. A small area of the epidermis showing multiple small vesicular lesions was scraped off with a scalpel. The collected material was ground with silicate in a mortar, suspended in saline, and purified by differential centrifugation.

Another suspension of vaccinia virus was prepared from glycerinated calf lymph vaccine as used for human vaccination. This had been held in stock for more than 6 months and was examined for the possible occurrence of morphological changes in virus treated in this manner. The vaccine was suspended in saline and purified as outlined above.

The preparations containing elementary bodies of varicella were obtained from four patients with typical clinical symptoms of chicken pox, one adult case and three children between 2 and 5 years of age. The fluids in these cases were collected with glass capillary tubes from unbroken vesicular skin lesions. The vesicle fluid from the adult patient was taken up with physiological saline and purified by differential centrifugation. Crusts which had formed, in the process of healing, on the pox lesions of this patient were removed, ground with silicate, suspended in saline, and purified as described above.

Only very small amounts of vesicle fluid were available from the children infected with varicella. The differential centrifugation, therefore, had to be abandoned, and the fluids were placed directly on the screens after they had been diluted with distilled water in proportions of approximately 1 in 10.

Nonspecific fluids and crust suspensions were obtained from vesicular epidermal lesions in man caused either by application of tincture of cantharides to the surface of the skin or by superficial burns. The specimens were prepared for electron microscopy in a manner similar to that used with the preparations from the adult patient infected with varicella.



FIGS 1-4 VARIOLA VIRUS FROM HUMAN CASE OF SMALLPOX

1 Magnification  $\times 24,800$

2-4 Shadowed with 22.1 mg of gold at the angle tangent 2/11 82  $\times 24,800$

FIGS 5-7 VACCINIA VIRUS FROM HUMAN CASE WITH SECONDARY VACCINIA

5 Magnification  $\times 24,800$

6-7 Shadowed with 24.5 mg of gold at the angle tangent 2/10 8  $\times 24,800$

FIG 8 VACCINIA VIRUS FROM CALF INFECTED WITH VACCINIA

Shadowed with 24.5 mg of gold at the angle tangent 2/10 8  $\times 24,800$

FIGS 9-10 VACCINIA VIRUS FROM GLYCERINATED SMALLPOX VACCINE

Shadowed with 22.4 mg of gold at the angle tangent 2/9 79  $\times 24,800$

at 2,500 rpm for 20 minutes. The elementary bodies were purified by a second centrifugation at 15,000 rpm for 1 hour. The sediment was finally resuspended in 0.9 ml distilled water, thus concentrating the suspension to one-tenth of its original volume. A small drop of the final suspension was then placed on the collodion-covered screens and dried in the usual manner.

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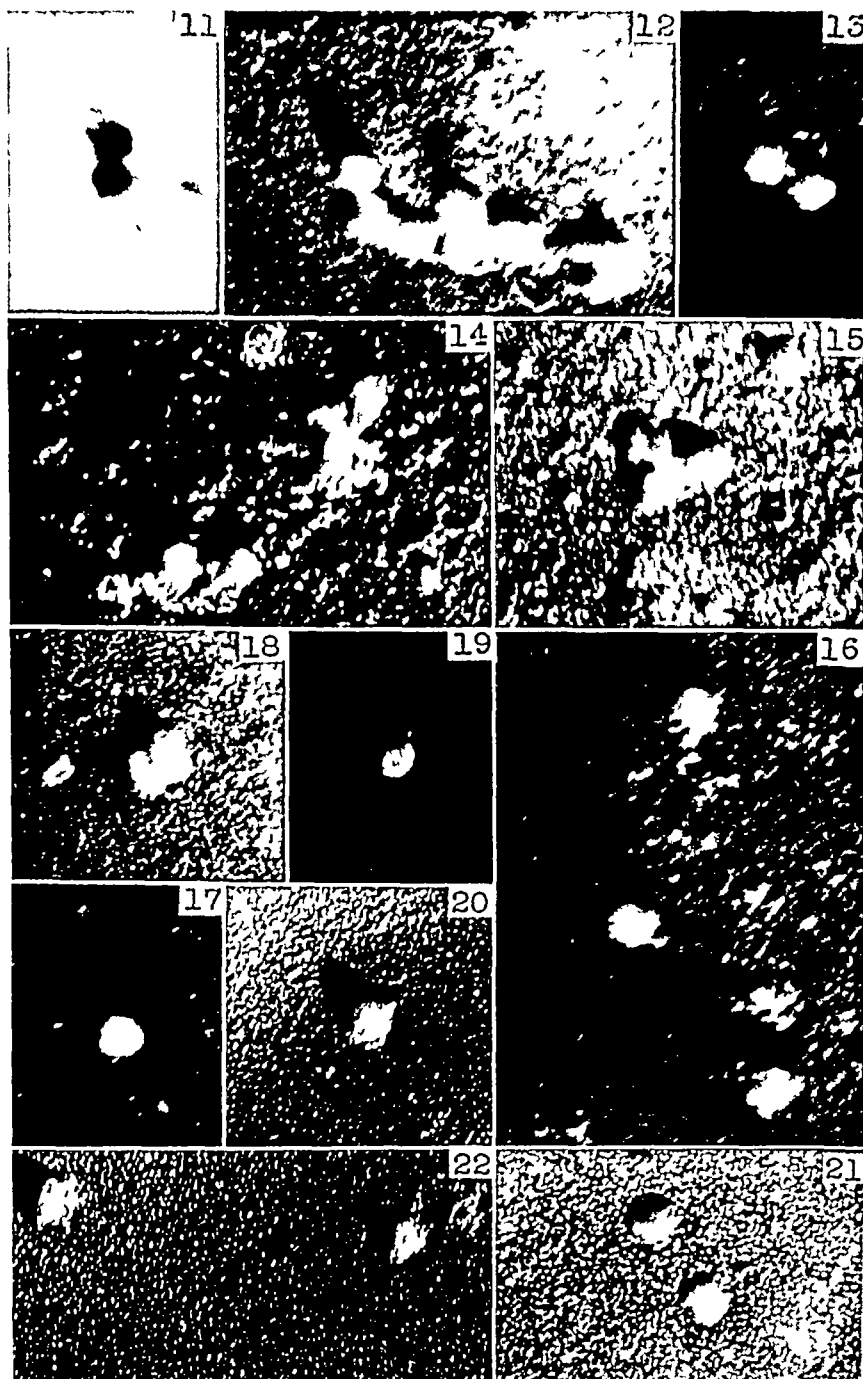
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The preparations containing elementary bodies of varicella were obtained from four patients with typical clinical symptoms of chicken pox, one adult case and three children between 2 and 5 years of age. The fluids in these cases were collected with glass capillary tubes from unbroken vesicular skin lesions. The vesicle fluid from the adult patient was taken up with physiological saline and purified by differential centrifugation. Crusts which had formed, in the process of healing, on the pox lesions of this patient were removed, ground with silicate, suspended in saline, and purified as described above.

Only very small amounts of vesicle fluid were available from the children infected with varicella. The differential centrifugation, therefore, had to be abandoned, and the fluids were placed directly on the screens after they had been diluted with distilled water in proportions of approximately 1 in 10.

Nonspecific fluids and crust suspensions were obtained from vesicular epidermal lesions in man caused either by application of tincture cantharides to the surface of the skin or by superficial burns. The specimens were prepared for electron microscopy in a manner similar to that used with the preparations from the adult patient infected with varicella.





FIGS 11-19 VARICELLA VIRUS FROM HUMAN CASES OF CHICKEN POX

11 Magnification  $\times 24,800$

12-13 Shadowed with 21.4 mg of gold at the angle tangent 2/9 79  $\times 24,800$

14-15 Shadowed with 23.0 mg of gold at the angle tangent 2/9 79  $\times 24,800$

16 Shadowed and magnified as figures 12 to 13

17 Shadowed with 21.2 mg of gold at the angle tangent 2/9 79  $\times 24,800$

18 Shadowed with 21.5 mg of gold at the angle tangent 2/11 82  $\times 24,800$

19 Shadowed with 21.2 mg of gold at the angle tangent 2/9 79  $\times 24,800$

FIGS 20-22 PARTICLES FROM NONSPECIFIC LESIONS (SEE TEXT)

Shadowed with 21.8 mg of gold at the angle tangent 2/9 79  $\times 24,800$



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# FURTHER STUDIES ON THE ALCOHOL TOLERANCE OF YEAST ITS RELATIONSHIP TO CELL STORAGE PRODUCTS

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It has been demonstrated earlier (Gray, 1946) that it is possible to increase the glucose tolerance of a yeast by a process of acclimatization involving daily transfer of the yeast into fresh medium of relatively high glucose concentration, i.e., 20 per cent glucose medium. It was found, however, that while the glucose tolerance of such an acclimatized yeast was increased, the alcohol tolerance was decreased—a rather unexpected finding in view of the previous finding that a yeast that has a high glucose tolerance normally exhibits a high tolerance for alcohol. Since the concentration of glucose that exerted an inhibitory effect upon yeast was found to be approximately the same concentration that induced plasmolysis of the yeast cell (Gray, 1945), it is obvious that some change in cell contents must have been induced during the acclimatization process that resulted in an increased capacity of the cell for glucose toleration. It is the aim of the present work to determine whether the concentrations of certain cell constituents bear any relationship to the capacity of a yeast for toleration of alcohol.

## MATERIALS AND METHODS

The yeast strains employed in this study were nos. 1, 3, 20, 22, 28, and 31 of the Seagram yeast stock culture collection. Of these yeasts all except no. 20 (*Willha anomala* Steuber) were strains of *Saccharomyces cerevisiae* Hansen. These particular strains were selected since they exhibit a wide range of alcohol tolerances, ranging from very low (no. 20) to very high (no. 31).

In all experiments the medium for yeast growth consisted of 0.7 per cent Difco yeast extract, 0.5 per cent  $\text{KH}_2\text{PO}_4$ , and 10 per cent glucose, the reaction of the medium being adjusted to pH 4.4 to 4.6 with normal  $\text{H}_2\text{SO}_4$ . The medium used for alcohol tolerance determinations was also prepared with Difco yeast extract and  $\text{KH}_2\text{PO}_4$ , instead of with the glucose yeast water medium used in earlier studies (Gray, 1941). Yeast grown without aeration or agitation was incubated at 30°C, whereas aerated cultures were grown at room temperature, which varied from 24 to 31°C.

Yeast cells in sufficient quantity for analysis were obtained by growing them in 500-ml portions of medium in liter Florence flasks (without aeration) or in 350-ml portions in liter Kjeldahl flasks (aerated cultures). Cultures were aerated by passing air through a sterilized cotton filter and thence through a small-bore glass tube inserted in the medium almost to the bottom of the culture flask. Aerated yeast was incubated for 24 hours and nonaerated yeast for 72 hours. At the end of the incubation period the yeast cells were centrifuged from the

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culture medium, resuspended in  $M/15$   $KH_2PO_4$ , and centrifuged again, the cells were then resuspended in fresh  $KH_2PO_4$  solution, centrifuged again, and killed with boiling distilled water. The killed cells were dried either in a vacuum oven at 65 C under a vacuum of 27 inches of mercury or in a drying oven without vacuum at 85 to 90 C.

The total cell carbohydrate content was determined by hydrolyzing the dried yeast in normal HCl as described by Smedley-MacLean (1922), filtering, washing, combining the filtrate and washings, and determining sugar as glucose by the method of Stiles, Peterson, and Fred (1926).

Total ether-soluble material, hereinafter referred to as fat or lipid, was determined by extracting dried, hydrolyzed yeast with anhydrous ether for 24 hours in a microsoxhlet apparatus and weighing the ether-soluble extract.

Alcohol-absorbing capacity was determined by centrifuging out the yeast cells and suspending 4 or 5 grams of wet yeast in 50 ml of approximately 5 per cent ethyl alcohol. At the end of 20 minutes, the cells were centrifuged from the alcohol solution and 25 ml of the supernatant were distilled to a 50-ml volumetric

TABLE 1

*A comparison of the fat and carbohydrate contents of acclimatized and nonacclimatized cultures of yeast no 1*

YEAST TYPE	AVERAGE TOTAL FAT CONTENT			AVERAGE TOTAL CARBOHYDRATE CONTENT		
	No of analyses	Grams per billion cells	Percentage of dry weight	No of analyses	Grams per billion cells	Percentage of dry weight
Acclimatized	13	0.00327	5.86	14	0.01079	19.14
Nonacclimatized	8	0.00244	3.70	9	0.01639	23.52

flask. The distillate was then made to volume with distilled water and the alcohol percentage determined by means of a Bausch and Lomb immersion refractometer. The amount of alcohol absorbed by the yeast cells was then calculated from the difference in alcohol concentrations of the initial alcohol solution and that in which the yeast cells had been suspended.

In all experiments, yeast cells used for inoculum were cells taken from 24-hour-old cultures.

## RESULTS

For the preliminary studies fat and carbohydrate determinations were made on acclimatized and nonacclimatized cultures of yeast no 1. Since it was found that acclimatized yeast cells weighed less than nonacclimatized cells (0.05623 g per billion cells as compared with 0.06546 g per billion for nonacclimatized cells), the results of these analyses are reported both as percentage of dry weight and on a per billion cell basis. The results of these initial analyses are shown in table 1.

From the results of table 1 it is evident that the amounts both of fat and carbohydrate stored by the cell were altered during the acclimatization process,

the total carbohydrate content being reduced whereas the total fat content was very markedly increased. In view of these results it was thought that a comparison of the fat and carbohydrate contents of several yeasts known to tolerate alcohol to different degrees might give some indication as to whether or not some orderly relationship exists between the amounts of these constituents stored by the cell and the latter's capacity to tolerate alcohol. Accordingly, analyses were made on yeasts 3, 20, 22, 28, and 31, the cells subjected to analysis being grown without aeration. The results, together with the results of analyses made on nonacclimatized yeast no. 1, are shown in table 2.

The analytical results in table 2 show that, in general, the higher the alcohol tolerance of the yeast, the less fat and carbohydrate are stored by the cell. With respect to cell fat storage a very close relationship apparently exists between this process and alcohol tolerance, thus, we see from column 2 that alcohol tolerance is inversely proportional to cell fat content. The yeasts used in these studies may be arranged as follows with respect to their alcohol tolerances:

TABLE 2  
*Fat and carbohydrate contents of yeasts of various alcohol tolerances*

YEAST NO	AVERAGE FAT CONTENT (% BY WEIGHT)	AVERAGE CARBOHYDRATE CONTENT (% BY WEIGHT)
20	6.84 (4)	24.27 (4)
22	6.29 (4)	26.67 (4)
1	3.70 (8)	23.52 (9)
3	3.31 (3)	16.95 (3)
28	2.94 (4)	17.02 (5)
31	2.53 (4)	15.62 (4)

Grown without aeration. Numbers in parentheses after fat and carbohydrate values indicate the number of analyses made. The yeasts are arranged in the table in the order of increasing alcohol tolerance.

31 > 28 > 3 > 1 > 22 > 20. On the basis of cell fat contents, the arrangement would be the exact reverse, i.e., 20 > 22 > 1 > 3 > 28 > 31. In general, the lower-alcohol-tolerant yeasts (nos. 20, 22, and 1) contain greater amounts of stored carbohydrate than do those yeasts of higher alcohol tolerance (nos. 3, 28, and 31). However, the close relationship that exists between alcohol tolerance and fat content is not exhibited between alcohol tolerance and carbohydrate content. Thus, on the basis of carbohydrate content, the yeasts would be arranged 22 > 20 > 1 > 28 > 3 > 31.

Since the foregoing results indicated that cell fat content was apparently related to alcohol tolerance, it was felt that if the fat content of a yeast could be altered by some means other than continued passage into high glucose medium and alcohol tolerance studies made on the yeast of altered fat content, additional information concerning this relationship could be obtained. Smedley-MacLean (1922), Smedley-MacLean and Hoffert (1923), and Nageli and Loew (1878) have pointed out that, when they are aerated, yeast cells store greater amounts of fat

The various strains of *S cerevisiae* (nos 1, 3, 22, 28, and 31) were grown with continuous aeration, and it was found that in each instance the yeasts stored larger amounts of fat than when grown without aeration. The results of analyses made on yeast from representative experiments of this type are presented in table 3.

Since with every yeast strain aeration during growth resulted in an increase in the amount of stored fat, partial proof of a determinative relationship between cell fat content and alcohol tolerance should be obtained through a comparison of the alcohol tolerances of aerated and nonaerated cultures of the same strain. Accordingly, an experiment was run in order to determine the respective capacities of aerated and nonaerated cultures of yeast no 1 for tolerating alcohol. Cultures were grown with and without aeration for 24 hours, at the end of which period cell counts were made. The cells were then centrifuged out of each culture and resuspended in sterile M/15  $\text{KH}_2\text{PO}_4$  in quantities that resulted in equal cell counts in the two suspensions. One-ml portions of these suspensions were then used to inoculate 24-ml portions of medium in 50-ml Erlenmeyer flasks. The

TABLE 3  
*The effect of aeration upon cell fat content of various strains of S cerevisiae*

EXPT NO	YEAST NO	AERATION ML AIR/350 ML CULTURE/MINUTE	FAT CONTENT (%)	
			Aerated	Nonaerated
I-44	22	14.8	7.74	6.29
I-56	1	14.9	4.18	3.70
I-45	3	17.0	6.11	3.31
I-37	28	15.9	6.27	2.94
I-36	31	10.0	9.15	2.53

initial glucose content of the medium was approximately 1 per cent and the initial alcohol content varied from 0 to 12 per cent by volume. After 24 hours' incubation at 30 C, glucose determinations were made, and the percentage of glucose utilization was calculated. The results of this experiment are presented in table 4.

Table 4 demonstrates that, at the highest alcohol concentration (12 per cent), the alcohol tolerance of the nonaerated yeast was somewhat higher than that of the aerated yeast. At the lower alcohol concentrations glucose utilization was of about the same order of magnitude for the two yeasts, however, this may find possible explanation in the fact that the initial cell count was rather high and also in the fact that with this yeast strain aeration did not result in so great an increase in fat content as in some of the other yeast strains employed.

A similar alcohol tolerance experiment was performed with aerated and nonaerated cultures of yeast no 31. As may be seen from the results in table 5, aerated cells of no 31 behaved in the same fashion as aerated cells of no 1, i.e., their alcohol tolerance was less than that of nonaerated cells.

Several experiments were run in order to determine whether the differences in



TABLE 1

*The effect of ethyl alcohol concentration upon glucose utilization by aerated and nonaerated cultures of yeast no 1*

CULTURE METHOD	INITIAL ALCOHOL (% BY VOLUME)	FINAL GLUCOSE (g/100 ML)	GLUCOSE UTILIZED (%)
Aerated	0 0	0 0266	97 53
Nonaerated	0 0	0 0223	97 93
Aerated	4 0	0 0284	97 36
Nonaerated	4 0	0 0269	97 50
Aerated	8 0	0 0315	97 07
Nonaerated	8 0	0 0260	97 58
Aerated	12 0	0 7600	29 43
Nonaerated	12 0	0 5950	44 75

Initial glucose content—1 077 g/100 ml, initial cell count in fermenters— $4 \times 10^6$ /ml

TABLE 5

*The effect of alcohol concentration upon glucose utilization by aerated and nonaerated cultures of yeast no 31*

CULTURE METHOD	INITIAL ALCOHOL (% BY VOLUME)	FINAL GLUCOSE (g/100 ML)	GLUCOSE UTILIZED (%)
Aerated	0 0	0 0380	96 61
Nonaerated	0 0	0 0381	96 59
Aerated	8 0	0 0623	94 44
Nonaerated	8 0	0 0575	94 86
Aerated	10 0	0 5040	55 00
Nonaerated	10 0	0 3470	69 01
Aerated	12 0	0 9900	11 61
Nonaerated	12 0	0 9460	15 54

Initial glucose content—1 120 g/100 ml, initial cell count in fermenters— $2 \times 10^6$ /ml

TABLE 6

*The effect of aeration on ethyl alcohol absorption by yeast cells*

YEAST NO	GRAMS OF YEAST USED	CULTURE METHOD	TOTAL G OF ALCOHOL ABSORBED	GRAMS OF ALCOHOL AB- SORBED/100 G OF YEAST
1	5	Nonaerated	0 073	1 460
1	5	Aerated	0 023	0 660
1	4	Nonaerated	0 057	1 425
1	4	Aerated	0 016	0 420
20	5	Nonaerated	0 075	1 500
20	5	Aerated	0 051	1 020
31	5	Nonaerated	0 079	1 580
31	5	Aerated	0 017	0 330

alcohol tolerance between aerated and nonaerated cultures of the same strain of yeast might not be due, at least in part, to differences in permeability of the

yeast cells to alcohol. Alcohol-absorbing capacities were taken as indicative of permeability to alcohol, and the results obtained with yeasts 1, 20, and 31 from studies of this nature are presented in table 6.

The results in table 6 show clearly that aerated cultures of yeasts 1, 20, and 31 all absorb less ethyl alcohol than nonaerated cultures, a fact which seems to indicate that aerated cells are less permeable to ethyl alcohol than are cells grown without aeration. Whether this change in permeability is due to changes in total lipid content that always accompany aeration or whether it is a change that occurs independently of change in lipid content has not been determined.

#### DISCUSSION

Fat and carbohydrate analyses made on nonaerated cultures of five strains of *S. cerevisiae* and *Willha anomala* demonstrate that for these six yeasts there is an obvious relationship between alcohol tolerance and quantity of cell storage products—a relationship which had been indicated by comparison of the alcohol tolerances and fat contents of acclimatized and nonacclimatized cultures of yeast no. 1. In general it may be said that yeasts of low alcohol tolerance store larger quantities of fat and carbohydrate than do yeasts of high alcohol tolerance. Thus, no. 20, a yeast of very low tolerance, was found to contain 24.27 per cent carbohydrate (calculated as glucose) and 6.84 per cent fat, as compared with 15.62 per cent carbohydrate and 2.53 per cent fat found in yeast no. 31, a yeast of high tolerance. In the yeasts studied, fat content varied inversely with alcohol tolerance, a condition which indicated a closer relationship between cell fat content and alcohol tolerance than between cell carbohydrate content and alcohol tolerance. The fact that cells of high fat and carbohydrate content have lesser capacities for toleration of ethyl alcohol is not surprising in view of the findings of Lindegren (1945a), who states that carbohydrate and fat reserves hinder the respiratory, fermentative, and budding activities of the cell. This same worker in another paper (Lindegren, 1945b) states that cells containing abundant accumulations of reserve material are in a state of dormancy, but that the dormancy is broken in a few hours when the cells are brought into a complete nutrient medium.

The fact that aerated and nonaerated cultures of the same yeast strain exhibit differences in total fat content as well as alcohol tolerance lends further weight to the view that fat content is at least in part a contributing factor to the cells' capacity for toleration of alcohol. Preliminary experiments on the effect of aeration on capacity for alcohol absorption yielded results which indicate that aerated yeast cells are less permeable to alcohol than are nonaerated cells, a phenomenon which may or may not in the present instance be directly related to the fat content of the cell. On the other hand, a possible explanation of the lower alcohol tolerance exhibited by aerated yeast cells may be sought in the observation of Lindegren (1945b) that alcohol causes many of the fat globules of the cell to coalesce and that dead cells also contain coalesced fat globules. Thus it may be that coalescence of fat globules, certainly a departure from the normal condition, simply occurs more readily in cells of high fat content. For a

complete explanation of the relationship between fat content and alcohol tolerance further studies are obviously necessary

#### SUMMARY

Analyses of the carbohydrate and fat contents of five strains of *Saccharomyces cerevisiae* and *Willia anomala* revealed that yeasts of high alcohol tolerance contain smaller amounts of these storage products than do yeasts of low alcohol tolerance. In the yeasts studied, fat content varied inversely with alcohol tolerance. Yeast no. 20, the least tolerant, contained the greatest percentage of fat, yeast no. 31, the strain of highest tolerance, contained the smallest percentage of fat, the other strains were intermediate between these two extremes.

Acclimatized cultures of yeast no. 1, known to be less tolerant of alcohol than nonacclimatized cultures, were found to store larger quantities of fat.

Aerated cultures of yeast, which always contain more fat than do nonaerated cultures, were found to be less tolerant of alcohol than anaerobically grown cultures of the same strain.

Preliminary experiments on alcohol-absorption capacities of aerated and non-aerated yeast indicate the possibility of a relationship between alcohol tolerance, fat content, and permeability of the cell to alcohol.

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#### DISCUSSION

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# PHYSIOLOGICAL STUDIES ON SPORE GERMINATION WITH SPECIAL REFERENCE TO *CLOSTRIDIUM BOTULINUM*<sup>1</sup>

## I DEVELOPMENT OF A QUANTITATIVE METHOD

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By far the majority of studies hitherto made on germination of bacterial spores have employed the appearance of visible turbidity as the criterion of germination. Obviously this technique can reveal no quantitative characteristics of the germination process and is therefore of value only in establishing that some germination does or does not take place. Even here its value may be questioned, since it has been clearly demonstrated that germination of spores of various organisms may occur without significant subsequent vegetative proliferation (Itano and Neill, 1919, Knight and Fildes, 1930, Knaysi, 1945, Knaysi and Baker, 1947). Furthermore, various environmental conditions imposed upon germinating spores may have no influence on the germination time but yet may alter appreciably the rate of subsequent vegetative development (Evans and Curran, 1943). Our own experiments confirm this finding.

Direct microscopic counts have been used for quantitative studies of the germination of aerobic spores (Eckelmann, 1918, Curran, 1931), but such a procedure is unduly wearisome and not readily adapted to use with anaerobes. Also, with certain species, for example, *Bacillus anthracis*, it may be very difficult to establish microscopically a criterion of germination, as noted by Fiscoeder (1909), Swann (1927), and Cook (1932).

The outstanding physiological difference between spores and vegetative cells of any one organism, namely, heat lability of the latter at a temperature innocuous to the former, has long been employed in quantitative approaches to spore germination, since it is assumed traditionally that when a spore cell is so changed that it becomes heat-labile, germination has taken place (Weil, 1901, Fiscoeder, 1909, Evans and Curran, 1943). Although Curran and Evans (1937, 1945b) have indicated that the heat-labile state may actually precede rupture of the spore wall and that some morphological changes characteristic of germination may occur prior to the loss of thermal resistance of the spore, heat differentiation of the germinated vs. the ungerminated spore appears to be the most practicable approach. The fact that a definite reproducible standard end point may be selected, viz., survival at a definite temperature for a definite period of time, even though somewhat arbitrary, outweighs the overlapping

<sup>1</sup> This project has been undertaken in co operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views of or being endorsed by the War Department.

between the physiological and morphological characters which renders the germination process an indistinct one

Consideration of other possible criteria of germination has repeatedly brought us to the conclusion that changes in heat lability are best from every point of view, and they form the basis of this work. The technique has been designed especially for *Clostridium botulinum*, an important organism in food poisoning, on which apparently no quantitative germination studies of any sort have been made. The method has been applied, with appropriate modifications, to other anaerobic species

#### EXPERIMENTAL

Some of the preliminary work was carried out with *C. botulinum* strain 115B, strain 62A was utilized in most of the work. Both strains were obtained from National Canner's Association and were repurified by isolation of colonies from serial shake tubes. Toxin formation was demonstrated for strain 62A by the fact that 1.0 ml of a Seitz filtrate of a 10-day broth culture was lethal for a guinea pig in less than 21 hours, whereas a control animal receiving 1 ml of the filtrate inactivated at 80 C for 15 minutes survived the observation period of 3 weeks.

Spore suspensions were prepared from 15-day cultures in Difco brain heart infusion broth with BBL thioglycolate supplement added. After four washings the cells were heated to 75 C for 30 minutes to destroy vegetative forms and then diluted in sterile distilled water containing glass beads. The final suspensions were shaken 1 hour on a rotary shaking machine to break up clumps and then stored in the refrigerator. The efficacy of the homogenization procedure was shown by repeated comparisons of counts by planting procedures and direct microscopic counts using the Petroff-Hauser chamber. The former averaged about 50 per cent of the latter, which may be considered fairly good correlation.

Other anaerobes used were *Clostridium chauvei*, *Clostridium histolyticum*, *Clostridium perfringens*, and the well-known food spoilage organism designated as putrefactive anaerobe no. 3679.

*The dormancy problem* The phenomenon of dormancy or delayed germination has presented a formidable experimental difficulty, which doubtless has been largely responsible for the lack of any really quantitative studies on spore germination in *C. botulinum*. Thus various authors report germination occurring only after incubation periods ranging from 53 days to 5½ years (Burke, 1919, 1923, Starn, 1924, Weiss, 1921, Sommer, 1930, Dozier, 1924, Dickson *et al*, 1922, 1925, Esty and Meyer, 1922, Dickson, 1928). Dormancy is not restricted to *C. botulinum* spores. It has been established for spores of other clostridia (McCoy and Hastings, 1928), for spores of aerobic species (Burke *et al*, 1925, Magoon, 1926, Morrison and Rettger, 1930a, 1930b), and even for cells of *B. coli* (Burke *et al*, 1925). Vegetative cells of *C. botulinum* have been observed to exhibit a degree of dormancy roughly comparable to that shown by the spores (Starn, 1924).

The prime requisite for systematic quantitative germination studies on "bot" spores is the complete elimination of the dormancy that has handicapped virtu-

ally all previous studies with this organism in this connection. Acting on the belief that cultural environment probably conditions dormancy, we felt that the germination medium offered the best prospects for our objective. This had been shown to be the case for aerobic sporeformers in which dormancy could be eliminated by supplying the correct medium (Morrison and Rettger, 1930a, 1930b, Curran and Evans, 1937). In recent years improvements have been made in media, which now give much higher counts than were possible with the media formerly used, a fact which indicates a high degree of success in eliminating the extremely long incubation period during which the spore count as measured by colony development would continue to increase.

Pork infusion thioglycolate medium<sup>2</sup> has been found to give considerably higher counts on a given spore suspension than do other media ordinarily used for such studies, but no indication is available that maximum counts are obtained in short enough time to be a useful tool for routine studies (Williams and Reed, 1942). Even this medium could be enhanced remarkably (30-fold) in the total count of heated spores obtainable after 23 days by the addition of 0.1 per cent soluble starch directly to the germination medium (Olsen and Scott, 1946).

Our work confirms both the superiority of pork infusion as a germination medium and the striking effect of starch on the germination process, and consequently on the spore counts. We have further demonstrated that starch acts primarily to adsorb and thus render inactive small amounts of substances, present in all media, that repress spore germination. This study is the subject of a separate paper. Finally, the spore-counting procedure ultimately evolved seemingly has eliminated dormancy as a practical obstacle in quantitative germinative studies on "bot" spores and, furthermore, for the first time enables maximum counts to be obtained in an incubation period no longer than that required for sizable colony development of any anaerobe, namely, 3 days.

*Comparison of several media popularly employed for counting C. botulinum spores.* One spore suspension heated to 75 C for 30 minutes to destroy vegetative cells was serially diluted in triplicate in the various agar media in flat Prickett counting tubes and incubated at 37 C. An effective anaerobic seal was obtained by covering the solidified agar with 3 to 4 ml of 2 per cent agar containing BBL thioglycolate supplement. Prickett tubes are essentially flattened test tubes and, though not generally used, have the decided advantage of permitting colony counts to be made in a thin layer of agar instead of in the entire diameter of a test tube. If maximum spore counts obtained in the pork thioglycolate starch

<sup>2</sup> The following procedure for preparation of this medium was kindly furnished by Dr. J. Yesair of National Canner's Association.

One pound of finely chopped lean pork is added to a liter of distilled water and boiled 1 hour. After removal of the meat and fat the filtrate is adjusted to pH 7.4 and to each liter are added 5 g of peptone, 1.6 g of tryptone, 1 g of glucose, 1.25 g of  $K_2HPO_4$ , and 15 g of agar.

It is our practice to omit the glucose and add 5 g of BBL thioglycolate supplement and 1 g of soluble starch per liter. We also adjust the final medium to pH 7.4. The troublesome precipitate resulting on boiling the final medium may be removed by filtration under negative pressure or discarded after decanting the supernatant.

medium are represented as 100, pork medium without starch gave 75, Difco brain heart infusion 55 to 60, brain heart plus 25 per cent peptone (Bristol, 1925) 45, BBL anaerobic agar in Brewer anaerobic dishes as employed by Curran and Evans (1946) 28, Difco liver veal 20, and Wilson and Blair's (1925) agar 5. Not only were counts consistently maximum in pork medium with thioglycolate starch supplement, but they occurred much earlier, reaching the peak in 3 days' incubation as compared to about 3 weeks for brain heart infusion agar. Actually counts could be made on the second day, but the colonies at this point are really too small to count easily or accurately. Three-day-old colonies provide no difficulties. If dormancy exists at all in the pork starch medium, it is believed to be a negligible interference in quantitative studies. Counts, i.e., colonies originating from germinating spores of *C. botulinum*, have never been observed to increase appreciably on prolonged incubation up to 2 to 3 weeks, whereas with other media the results are meaningless before that time. Counts of spore suspensions have regularly been around 50+ per cent of direct microscopic counts (Petroff-Hauser chamber), a not too unsatisfactory correlation considering the tendency of the spores to clump in various degrees and the fact that viability doubtless is not 100 per cent. A special experiment to detect dormant spores (i.e., ungerminated viable spores) in this medium after 3 days' incubation failed to reveal any that germinated up to the time of writing, a period of 4½ months. Typical dormancy under these conditions manifests itself as gradually increasing counts over the entire incubation period.

*Detailed procedure for studying germination of C. botulinum spores* The following procedures typify our approach to the quantitative study of the germination process and of factors influencing it. One-ml portions of the appropriately diluted spore suspension were transferred to tubes containing 9 ml of Difco brain heart infusion broth with BBL thioglycolate supplement. This particular germination medium was chosen because the relatively moderate rate of germination occurring in it allows the study of factors both stimulatory and inhibitory to germination. The tubes were then heated to 75 C for 20 minutes to expel dissolved oxygen and to effect any possible "heat activation" of the spores (Evans and Curran, 1943, Curran and Evans, 1945a). After appropriate intervals of incubation at 37 C in air or other atmosphere, replicate (usually triplicate) tubes were reheated to 75 C for 20 minutes to destroy any vegetative cells that had developed as a result of germination. Residual spore counts were made as above in pork thioglycolate starch agar. Available data indicate that germination is somewhat faster at 30 C than at 37 C, but for convenience 37 C was used in all these germination studies.

*Expression of results* Most workers have utilized absolute numbers of residual spores as a basis for interpreting the effect of a particular treatment on the germination process. We feel that *percentage* of germination is to be preferred as a more reliable basis for interpretation of results, because of the large populations employed, and especially is this true when germination is largely complete. For example, on the comparative basis of residual, relatively small spore counts, Evans and Curran (1943) concluded that a considerable accelera-



tion of germination of aerobic spores had resulted from preheating the spores in glucose broth. If, however, the residual spores are considered as a fraction of a large population and calculated as percentage of that population, the stimulatory effect for 1 out of the 7 positive cases would be less than 6 per cent and in one instance less than 0.1 per cent. Certainly the magnitude of the effect is much different when expressed percentagewise, the only valid way in our estimation. A simple numerical example of this point seems worth while. Suppose a germination test is run under two treatments on a spore suspension containing 5,000 spores per ml and the residual spore count shows 100 and 200 per ml, respectively. While the 100 per cent difference between the residual spore counts seems striking, the values for *germinated* spores are the design of the experiment and the more important data. These would be 4,900 and 4,800, respectively, or 98 and 96 per cent germination, an insignificant difference in work of this nature.

*Application to germination under stimulatory and under inhibitory treatments*  
Though germination curves may be employed for determining the effect of a given factor throughout the time course of germination, the effect taken at any one significant incubation time is usually sufficient.

TABLE 1  
*Effect of starch on germination*

STARCH	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION (%)
	<i>hours</i>			
0.1%	0	575		
	24	400	175	30
	24	60	515	90

If a stimulatory factor is being studied, the time selected should be such that germination is relatively small in the control in order to allow the treatment to manifest itself to the maximum. An example is the effect of 0.1 per cent soluble starch in the germination medium (brain heart broth) shown in table 1. The spore-counting medium was the usual pork thioglycolate starch agar.

On the other hand, an inhibitory effect is best demonstrated at an incubation time when germination is nearly maximum in the controls. Table 2 demonstrates that germination in brain heart broth is considerably retarded by momentary contact with air during the removal of sample tubes for counting from a desiccator made anaerobic with an inert gas phase (natural gas,  $\text{CH}_4$ ), even though re-exhaustion with a Hyvac pump and replacement with inert gas is done without delay. In the unopened desiccator 87 per cent of the spores germinated, whereas in the desiccator opened briefly at 20 and 24 hours only 29 per cent germination was obtained—a striking inhibition.

*Accuracy and reproducibility of spore counts* The degree of accuracy obtainable with this method depends, of course, on the number of replicates used for determining the "average" counts. For zero controls triplicate tubes were generally used, with triplicate dilutions plated for each tube, or a total of nine

counts For other averages triplicate tubes with duplicate or triplicate platings of dilutions were usually employed The over-all reproducibility and accuracy of counts on a *C botulinum* spore suspension stored in the refrigerator are illustrated in table 3

Agreement between replicate counting tubes seems to depend on several factors, including scrupulous chemical cleanliness of glassware, the presence of

TABLE 2

*Effect of momentary contact with air on germination in natural gas*

	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION (%)
	<i>hours</i>			
Desiccator unopened	0	560		
	28	74	485	87
Desiccator opened twice to remove samples at 20 and 24 hours	0	535		
	28	380	155	29

TABLE 3

*Reproducibility of spore counts on a single suspension*

DATE OF COUNT	SPORES PER ML
3/10	535
3/15	560
3/27	540
4/2	575
4/9	530
4/20	560
4/20	520
4/24	600
4/27	500
4/30	535
5/22	540
6/1	530
Average	548

*Extreme deviation from mean*

$$\frac{50}{548} = 9\%$$

soluble starch in the counting medium, and the atmosphere in which germination takes place

*Factors conducive to variability* Considerable evidence has been accumulated that germination of "bot" spores is extremely susceptible to minute amounts of substances in the general category of impurities As mentioned earlier, these occur in all organic media and possibly in tap water At any rate, a high order of variation was experienced between replicate tube counts of a given dilution of the suspension until a rigorous cleaning procedure was adopted A marked re-

duction in count variation followed when the cleaning was done with "dref," followed by thorough rinsing with distilled water. However, the best means of minimizing this tube to tube variation proved to be the additional feature of incorporation of the starch. The adsorption effect mentioned above explains this leveling action of the starch.

Finally, even though the cultivation of the germination tubes in an atmosphere of ordinary air gives good growth, use of an inert atmosphere of natural gas further reduced appreciably the count variation in replicate tubes.

These procedures work equally well with the four other anaerobic spore-formers tested.

#### SUMMARY

A simple, reasonably accurate method for quantitative study of spore germination in *Clostridium botulinum* and other anaerobes is described. The obstacle of dormancy has been eliminated, maximum counts appearing in 3 days. Illustrations are given of the application of the method.

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# PHYSIOLOGICAL STUDIES ON SPORE GERMINATION WITH SPECIAL REFERENCE TO CLOSTRIDIUM BOTULINUM<sup>1</sup>

## II QUANTITATIVE ASPECTS OF THE GERMINATION PROCESS

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Though virtually every aspect of the growth and death rates of bacterial cultures has been subjected to searching kinetic analysis, the spore germination process itself has been largely neglected. Probably this is due particularly to the failure to discriminate precisely between the actual germination process and the subsequent vegetative development and, especially with anaerobes, to inadequate counting methods. The only two reports really bearing on this issue, both dealing with aerobic sporeformers, failed to evaluate the process other than to conclude that the number of spores germinating increased with time (Fischoeder, 1909, Eckelmann, 1918). This paper deals quantitatively with the germination process in the anaerobic sporeformer *Clostridium botulinum* strain 62A.

### EXPERIMENTAL

The germination medium was Difco brain heart infusion broth with BBL thio-glycolate supplement. The counting medium, procedures, and other details are as described in the preceding paper (Wynne and Foster, 1948).

It was observed repeatedly that the logarithm of numbers of residual spores in a spore suspension in a germination medium plotted against time gives a straight line, at least until 95 per cent or more of the spores originally present have germinated.

Data for a typical experiment are given in table 1 and are plotted as curve A in figure 1 together with curves B and C, the latter two representing experiments in which the germination was allowed to take place in air, as contrasted to the natural gas atmosphere (CH<sub>4</sub>) in the experiment described by curve A and table 1. The general equation for a first order reaction may be written as

$$434K = 1/t \log \frac{I}{I - G} \quad (1)$$

in which  $K$  = a constant

$t$  = time elapsing since beginning of germination ( $t_0$ )

$I$  = no. spores per ml at beginning of germination

$G$  = germinated spores at time  $t$

<sup>1</sup> This project has been undertaken in co operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or having the endorsement of the War Department.

But since  $I - G = R$  (residual spores), we may substitute  $R$  in the equation above, giving

$$434K = 1/t \log I/R \quad (2)$$

or

$$K = \frac{(\log I - \log R)}{434t} \quad (3)$$

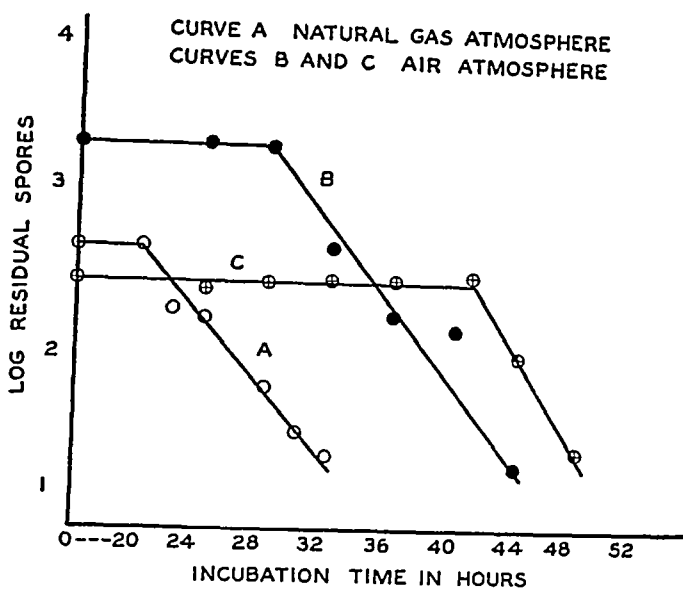


FIG. 1 GERMINATION CURVES OF *C. BOTULINUM* 62 A

TABLE 1  
*Germination in natural gas*

INCUBATION hours	AVERAGE COUNT RESIDUAL SPORES	GERMINATED SPORES	% GERMINATION	LOG RESIDUAL SPORES	K CALCULATED FOR $t_0 = 20$ HR
0	560			2.75	
20	560	0	0	2.75	
22	230	330	59	2.36	449
24	200	360	64	2.30	259
28*	74	485	87	1.87	254
30	39	520	93	1.59	267
32	28	530	95	1.45	250

\* Counts for 26 hours are not given owing to accidental overheating of the tubes in the incubator

The  $K$  values in table 1 were computed assuming  $t_0 = 20$  hours, which obviously is a minimum value as under the experimental conditions  $t_0$  could have been any time between 20 and 22 hours. Owing to this experimental inaccuracy the  $K$  value for the 22-hour period is off, but is reasonably constant for the other periods, indicating that the germination process conforms to a first-order reaction. Curve A, figure 1, shows also that the germination process is logarithmic, thus conforming to the kinetic picture typical of all growth and killing rates of bacteria.

TABLE 2  
*Relation of duration of lag to concentration of inoculum in air*

INOCULUM	LAG PERIOD	C, CALCULATED*
<i>Spores/ml</i>	<i>hours</i>	
2,400	28	95
2,100	28	93
530	36	98
340	40	101

\*Equation (4)

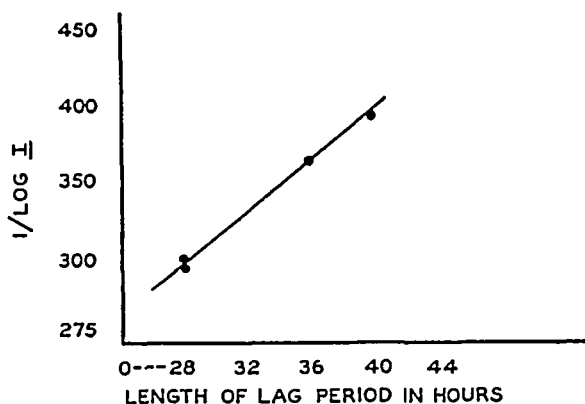


FIG 2 RELATIONSHIP OF LENGTH OF LAG PHASE OF GERMINATION TO [RECIPROCAL OF LOG OF NUMBER OF SPORES PER ML IN INOCULUM]

It is obvious from curves B and C in figure 1 that the duration of the lag period depends on the concentration of the inoculum. By extrapolating these logarithmic germination curves, as well as others, values representing the length of the lag period were obtained according to the inoculum density as given in table 2. The length of the lag period appears to vary as the reciprocal of the logarithm of inoculum numbers and obeys the following relation

$$L = \frac{C}{\text{Log } I} \quad (4)$$

in which  $L$  = length of lag phase in hours

$I$  = no. of spores per ml in inoculum

$C$  = a constant

The validity of this expression is borne out by the fact that the values of  $\frac{1}{\log I}$  plotted against  $L$  made a straight line (figure 2), computed values for  $C$  at the different levels of inoculum agree fairly well, as seen in table 2

These data were obtained from cultures incubated in ordinary air. Curiously, these relations did not apply when incubation was done in a desiccator with an atmosphere of natural gas, at least under these conditions

#### DISCUSSION

Neither Fiscoeder (1909) nor Eckelmann (1918) drew any conclusions pertaining to the kinetics of the germination observed by them, but a plot of their data against time shows they actually were concerned with logarithmic germination processes. However, data of the former author for the germination of *Bacillus anthracis* in goat and dog sera indicate that germination was not logarithmic under those conditions. It is obvious that any factor inimical to germination may at once rule out the logarithmic relation.

The tentative equation suggested above for the length of the lag period obviously should be tested further with more data. If this equation is valid, plotting the length of the lag phase against the logarithm of inoculum directly should give a hyperbola, since  $(L)(\log I) = C$ . Though such a plot from the values determined above is compatible with a hyperbolic curve, the points are too few to define the curve clearly.

In contrast to the paucity of information on the lag period of spore germination an abundance of work has been done on vegetative cells, but as spore germination does not involve actual cell multiplication we consider the spore problem one distinct from vegetative activity. The apparent failure of equation (4) to hold in an atmosphere of natural gas (i.e., no oxygen) cannot be explained at present. Suspicion might be directed to the influence of the O-R potential on germination, an effect found to apply to spores of the anaerobe *Bacillus tetani* (Fildes, 1929, Knight and Fildes, 1930). Rapidity of germination depended on the time required for the medium, and presumably the interior of the spores, to reach a suitable reducing intensity, and spore numbers might influence this action. Finally, natural gases may contain traces of impurities which might account for this discrepancy.

#### SUMMARY

The germination process of spores of *Clostridium botulinum* 62A is logarithmic. In air atmosphere the length of the lag period in germination varied inversely with the logarithm of the number of spores per ml in the inoculum. These relations are expressed mathematically



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# THE ENHANCEMENT OF PENICILLIN EFFECTIVENESS IN VIVO BY TRACES OF COBALT<sup>1</sup>

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It has been shown that the *in vitro* activity of dilute solutions of penicillin against *Staphylococcus aureus* may be increased 4- to 8-fold in the presence of trace amounts of cobalt chloride (Strait, Dufrenoy, and Pratt, 1947) and that the enhancing effect is even more pronounced when gram-negative, penicillin-resistant organisms such as *Eberthella typhosa*, *Proteus vulgaris*, or *Escherichia coli* are used as test organisms (Pratt and Dufrenoy, 1947b). This effect seems to be specific to cobalt among the ions studied, since salts of Ni, Mn, Pt, Ir, Fe, Zn, Sr, Cd, Li, Cu, Ag, Au, and Bi have been studied, and none has been so effective in producing a similar enhancement of penicillin activity in the range from 0.01 to 10 ppm.

A similar action of cobalt on penicillin activity has now been demonstrated in mice inoculated with lethal doses of *E. typhosa*.

Different groups of 30 adult mice (Swiss Klocke) were injected intraperitoneally with 0.5 ml aqueous solutions of crystalline sodium benzyl penicillin alone (2,000 units per animal) or with the same dose of the penicillin combined with different amounts of cobalt ranging from 4 to 256 micrograms of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  per animal, or with the solutions of cobalt chloride alone. Thirty minutes later the animals were inoculated intraperitoneally with 0.4 ml of a 6-hour broth culture of *Eberthella typhosa* (approximately  $2 \times 10^8$  organisms). This procedure is essentially similar to that proposed by Welch, Randall, and Price (1947) for the detection of an enhancing "factor" found in certain commercial lots of amorphous penicillin.

Experimental results are shown in table 1. Five experiments with the same procedure but with different control conditions all yielded similar results.

The data for 24 hours show that 64 micrograms of cobalt chloride administered simultaneously with 2,000 units of crystalline benzyl penicillin enhanced the activity of the penicillin approximately 50 per cent, i.e., 2,000 units with cobalt chloride protected animals for 24 hours as well as did 3,000 units without cobalt. Larger amounts of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  administered with the penicillin caused greater enhancement. Thus with 256 micrograms of cobalt chloride administered with 2,000 units of penicillin, the enhancement of the activity as seen from the 48-hour mortality data is of the order of 100 per cent, i.e., 2,000 units of penicillin plus 256 micrograms of cobalt chloride protected animals for 48 hours as well as

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<sup>2</sup> With the laboratory assistance of Virginia Lamb and Ellen Clark.

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did 4,000 units without cobalt. There were no further deaths after 48 hours. The percentage of enhancement at 24 hours was much greater than at 48 hours. Administration of cobalt chloride alone conferred no protection on the infected animals. Therefore, the result that was observed cannot be interpreted merely as the additive effect of two antibiotics acting simultaneously.

It seems likely that the schedule of injection that was used failed to provide a sufficient concentration of cobalt over a long enough period of time to sustain adequately the enhancing action. Copp and Greenberg (1941) showed that in rats small doses of intraperitoneally injected cobalt were excreted largely in the

TABLE 1  
*The effect of cobalt with penicillin*

TEST MATERIAL INJECTED 30 MINUTES BEFORE INOCULUM OF <i>E. TYPHOSA</i>	NUMBER OF MICE	PERCENTAGE OF MORTALITY AFTER	
		24 hours	48 hours
Control (inoculum only)*	555		90*
Control (inoculum only)	100	92	92
Penicillin (2,000 units)*	530		78*
Penicillin (2,000 units)	90	77	80
Penicillin (3,000 units)*	560		67*
Penicillin (3,000 units)	60	72	77
Penicillin (4,000 units)*	80		45*
{ Penicillin (2,000 units) plus $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.064 mg) }	60	69	79
{ Penicillin (2,000 units) plus $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.256 mg) }	90	27	39
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.256 mg)	60	95	96
Cobalt control $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.256 mg)† with no subsequent inoculation with <i>E. typhosa</i>	10	0	0

\* Accumulated data from Microbiological Research Department of Cutter Laboratories, Berkeley, California.

† No deaths occurred when doses as high as 0.512 mg per animal were administered, but the animals seemed drowsy and disinclined to move voluntarily for 24 hours, although they were active if prodded slightly. Higher doses were not used. No symptoms of toxicity or altered action were seen in animals receiving 0.256 mg.

first few hours. 70 per cent via the urine in the first 10 hours and approximately only an additional 10 per cent in the next 14 hours.

It was observed *in vitro* (Strait, Dufrenoy, and Pratt, 1947) that there is an optimum concentration of cobalt for securing the maximum enhancement of penicillin activity and that to secure the best effect a period during which the cobalt is in contact with the pathogen before the application of penicillin is desirable. Thus it may be surmised that, *in vivo*, appropriate doses of cobalt chloride administered before and periodically for several hours after appropriate doses of penicillin would be effective in increasing and prolonging the effect of the penicillin. We are investigating this matter as well as the use of cobalt-penicillin mixtures in the treatment of acutely and chronically infected animals.

The enhancement of penicillin activity effected by traces of cobalt *in vitro* and *in vivo* differs from that induced by the "factor" reported by Welch, Randall, and Price (1917), which is detectable only *in vivo*

These experiments were conceived because of the demonstrated effect (Albert, 1917) of cations on the bacteriostatic activity of agents with which they had the capacity to form complexes. Evidence has been presented (Dufrenoy and Pratt, 1917, Pratt and Dufrenoy, 1917a) that agrees with the hypothesis that the bacteriostatic activity of penicillin can be correlated with a shift in the oxidation-reduction potential, possibly attributable to dehydrogenation of the —SH groups from sulfhydryl-containing proteins. Thus it may be proposed that the synergistic effect of cations may be ultimately associated with the formation of complexes with —SII-containing groups or with some other essential component of an energy-providing oxidation-reduction system. The degree of inhibition effects exhibited by the various cations may be related to the degree of binding of the cations in the complex. Thus Cd and Ag, which form stable complexes, are highly toxic, whereas Co, which forms loose complexes with —SH groups, is much less toxic.

#### SUMMARY

The enhancement of penicillin effectiveness by the addition of small amounts of cobalt, previously observed *in vitro*, has been demonstrated *in vivo*. Administration of 256 micrograms  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  with 2,000 units of crystalline sodium benzyl penicillin per animal exerted a protective action against *Eberthella typhosa* in adult mice equivalent to at least 4,000 units of the penicillin alone. Concentrations as low as 64 micrograms of cobalt chloride produced some enhancement of penicillin effectiveness. Cobalt chloride alone in these concentrations was not toxic and conferred no protection on the animals.

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# THE GRANULOSE REACTION OF CERTAIN ANAEROBES OF THE "BUTYRIC" GROUP

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McCoy, Fred, Peterson, and Hastings (1926) state "One of the most distinctive features of the group to which the butyl organism belongs is the granulose reaction. Young vegetative cells stain yellow with iodine. As sporulation approaches and the cells take on clostridial form, they store granulose and stain blue or violet with iodine. As the spores mature the granulose reaction is lost, indicating that the reserve material has been utilized in sporulation." This microscopic observation has long been used as a criterion for the classification of members of this iodophilic group. Batchelor and Curie (1929) have grown cultures of soil butyrics in oat jars, then flooded the plates with iodine, and determined the iodophiles by the macroscopic observation of the blue-black colonies.

In reporting a study of the cultivation and identification of the butyric anaerobes (Spray, 1937), there was discussed a striking phenomenon observed in semi-solid agar cultures of certain members of this group. This reaction was obtained when Gram's iodine was added to glucose semisolid agar cultures, which immediately turned dark violet. This reaction was consistently obtained with only certain members of the group and thus appears to have a possible value for the differentiation of certain species. In view of the chaotic taxonomy of the butyric group, such a "species" character may prove of the same significance as the "stormy fermentation" of *Clostridium perfringens*, the "iron-gelatin" reaction of *Clostridium histolyticum*, and the "vanillin-violet" (skatol) reaction of *Clostridium sporogenes* and *Clostridium parabotulinum* (Spray, 1936).

The conditions under which granulose is formed have been a matter of considerable study. We mention here only the study of Svartz (1930), who states, "The typical iodophil, clostridium-forming bacteria in the feces never deposited iodophil substance in a medium with a pH of less than about 6.6, even when there was plenty of carbohydrate in the medium." This held true also for a series of butyrics from various sources.

We have confirmed this statement in regard to a variety of butyrics obtained through the courtesy of Dr. Elizabeth McCoy and Dr. Ivan Hall—including McCoy's *Clostridium saccharobutyricum* (Wis 63), *Clostridium felsineum* (Wis 41), *Clostridium pasteurianum* (Wis 60), *Granulobacter saccharobutyricus-immobile-non-liquefaciens* (Wis 24), *Clostridium butyricum-iodophilum* (Wis 61), *Clostridium butylicus* (Wis 39), "unknown butyric" (Wis 33), *Clostridium acetobutylicum* (Wis ), *Clostridium roseum* (Wis 43), and a series of "unknown butyrics" from Dr. Hall, nos 9039, 9041, 3234, 7241, 3813, 274, 9040, 9042, and 9043. However, these facts did not seem to apply to certain strains, including

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*C. pasteurianum* (Wis 5), *Clostridium beijerinckii* (Wis 68), *C. beijerinckii* (ATCC 858), and Hall's "unknown butyrics" nos 1067, 1334, 3815, and 3235

TABLE 1

Estimation of growth determined by turbidity and gas formation at 17 hours' incubation

CULTURE	TUBE NO													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	GLUCOSE %													
	20	18	16	14	12	10	08	06	04	02	01	005	0025	000
Wis 5	4	4	4	4	4	4	4	3	2	1	1	±	?	—
Wis 60	4	4	4	4	4	4	4	3	2	1	1	±	?	?
Wis 24	4	4	4	3	2	2	2	2	2	1	1	±	±	?
Wis 39	4	4	4	4	4	4	4	3	2	1	1	±	?	?
Wis 68	4	4	4	4	4	4	4	4	3	2	1	±	?	?
Wis 61	4	4	4	4	4	4	4	3	2	1	1	1	±	?

TABLE 2

Granulose reaction at 17 and 41 hours' incubation by the spot plate iodine test

CULTURE		TUBE NO													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		GLUCOSE %													
		20	18	16	14	12	10	08	06	04	02	01	005	0025	000
No 5	17 hr	4	4	4	4	4	4	4	3	2	1	±	?	—	—
	41 hr	±	3	4	3	2	3	2	1	1	—	—	—	—	—
No 60	17 hr	—	—	—	?	?	—	—	—	—	—	—	—	—	—
	41 hr	—	—	—	—	—	—	—	—	—	—	—	—	—	—
No 24	17 hr	—	—	?	?	?	?	?	?	—	—	—	—	—	—
	41 hr	—	—	—	—	—	—	—	—	—	—	—	—	—	—
No 39	17 hr	—	2	?	2	3	±	±	?	—	—	—	—	—	—
	41 hr	—	?	?	—	—	—	—	—	—	—	—	—	—	—
No 68	17 hr	4	4	4	4	4	4	4	4	3	2	1	±	—	—
	41 hr	3	2	3	2	3	2	2	1	±	—	—	—	—	—
No 61	17 hr	—	—	?	?	?	—	?	—	—	—	—	—	—	—
	41 hr	—	—	—	—	—	—	—	—	—	—	—	—	—	—

We proceeded then to a more intimate study of the phenomenon, applying the iodine test to semisolid glucose agar cultures. A sugar-free base was prepared (1 per cent Difco neopeptone, 1 per cent Difco tryptone, and 0.25 per cent agar in tap water). To one-half of this was added 2 per cent glucose, and dilu-



tions were made with the sugar-free portion, giving glucose concentrations of 2.0, 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.025 per cent, and a sugar-free control. The final reaction after autoclaving in tubes was about pH 7.2.

These glucose dilutions were inoculated with selected strains from the group enumerated above, and all tubes were incubated at 37 C. After 17 hours' incubation all tubes were examined for presence and amount of growth and gas, as recorded in table 1. At the same time samples were aseptically removed with capillary pipettes and tested for granulose on a spot plate, 2 drops of Gram's iodine being added to 5 drops of culture and stirred. The darkest violet reaction

TABLE 3

*Beckman pH determinations and granulose reactions at 117 hours' incubation, iodine added to shaken tube cultures*

CULTURE		TUBE NO													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		GLUCOSE %													
		2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2	0.1	0.05	0.025	0.00
No 5	pH Grn	1.38 4	1.17 4	1.15 4	1.26 4	1.30 4	1.33 4	1.41 4	1.55 3	1.78 2	5.17 ±	5.70 —	6.04 —	6.30 —	6.69 —
No 60	pH Grn	5.08 —	5.05 —	5.04 —	5.06 —	5.09 —	4.82 —	4.73 —	4.78 —	4.92 —	5.32 —	5.79 —	6.24 —	6.45 —	6.72 —
No 24	pH Grn	5.06 —	5.04 —	5.10 —	5.01 —	4.95 —	4.86 —	4.75 —	4.80 —	4.94 —	5.34 —	5.88 —	6.23 —	6.45 —	6.69 —
No 39	pH Grn	5.10 —	5.14 —	5.04 —	5.18 —	5.20 —	4.78 —	4.78 —	4.80 —	4.87 —	5.43 —	5.92 —	6.27 —	6.46 —	6.76 —
No 68	pH Grn	4.61 4	4.64 4	4.68 4	4.69 3	4.64 4	4.66 3	4.71 3	4.89 2	5.00 ±	5.46 ?	6.02 —	6.48 —	6.55 —	6.74 —
No 61	pH Grn	5.07 —	5.05 —	5.11 —	5.02 —	4.97 —	4.90 —	4.81 —	4.83 —	4.93 —	5.36 —	5.86 —	6.24 —	6.46 —	6.68 —

was recorded as 4 (+++), and less color in terms of comparative intensity as 3, 2, 1, ±, and —. The spot plate test was repeated after 41 hours' incubation, and the results of the two series are presented in table 2.

Finally, after 117 hours' incubation the pH of each tube was determined by the Beckman potentiometer, after which the entire contents of each tube were tested for granulose by shaking up the culture and then adding from 5 to 25 drops of Gram's iodine to each tube. In this test an initial strong reaction was followed by rapid fading. Further additions of 5 drops delayed this fading, until with 25 drops (to about 7 ml of medium) a relatively stable color was developed.

An attempt was made to measure this color in rather definite terms of starch-

iodine color A 10 per cent solution of soluble starch (Difco) was accurately prepared in distilled water From this were prepared 10 dilutions and a starch-free control To tubes of these dilutions, in the same volume as the cultures, the same amounts of iodine were added and the colors recorded in similar terms These starch-iodine tubes, as color standards, were used for comparison with the culture reactions Incidentally, we found that the soluble starch gave a color

TABLE 4  
Soluble-starch-iodine reactions (standards)

TUBE NO	1	2	3	4	5	6	7	8	9	10	11
Soluble starch %	1 00	0 50	0 25	0 125	0 063	0 031	0 016	0 008	0 004	0 002	0 000
Starch-iodine reaction	4	4	4	4	4—	3	2	2—	1	?	—

TABLE 5

Granulose reactions at 117 hours' incubation recorded in terms of equivalent soluble starch iodine reactions (table 4)

CULTURE	TUBE NO													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
W <sub>18</sub> 5	0 5	0 5	0 50	0 25	0 25	0 25	0 125	0 063	0 016	0 002	0 00	0 00	0 00	0 00
W <sub>18</sub> 68	0 5	0 5	0 125	0 063	0 063	0 063	0 032	0 032	0 008	0 002	0 00	0 00	0 00	0 00
W <sub>18</sub> 60														
W <sub>18</sub> 24														
W <sub>18</sub> 39														
W <sub>18</sub> 61														

TABLE 6

Significant fermentations of 1 per cent carbohydrates in semisolid agar base

CULTURE	LACTOSE	CORN STARCH	DEXTRIN	GLYCOGEN	SALICIN	GLYCEROL	MANNITOL	SORBITOL	DULCITOL	INOSITOL
W <sub>18</sub> 5	—	—	—	—	—	+	+	+	—	+
W <sub>18</sub> 60	+	+	+	+	+	—	+	+	+	+
W <sub>18</sub> 68	+	—	—	—	+	—	+	+	—	+

reaction almost exactly comparable to that of the granulose Similar dilutions of cornstarch gave a distinctly bluer color, much less satisfactory for comparison The results of these tests are recorded in tables 3, 4, and 5, only for the selected significant cultures *C. pasteurianum* (W<sub>18</sub> 5), *C. pasteurianum* (W<sub>18</sub> 60), *Granulobacter saccharobutyricus-immobile-non-liquefaciens* (W<sub>18</sub> 24)—incidentally, a misnomer for this strain, both because of the name and the fact that the

culture is actually motile, *C. butylicus* (Wis 39), *C. beijerinckii* (Wis 68), and *C. butyricum-iodophilum* (Wis 61)

Of the other cultures enumerated above, and not included in the tables, the Wisconsin series 63, 41, 33, *C. acetobutylicum*, and 43, and Hall's "unknowns" 9039, 9041, 3231, 7241, 3813, 274, 9040, 9042, and 9043 fall in the granulo-negative group. The ATCC 858 and Hall's "unknowns" 1067, 1334, 3815, and 3235 fall in the granulo-positive group.

From these reactions of selected, representative species there appear three interesting observations: (1) the granulo reaction may be determined conveniently and sharply in a semisolid glucose agar, (2) this reaction is not necessarily inhibited by a strong acidity (as low as pH 4.15), and (3) the test by the method given seems to distinguish two species (*C. pasteurianum*, Wis 5, and *C. beijerinckii*, Wis 68) from all of the other butyric anaerobes tested.

It should be noted that *C. pasteurianum* (Wis 60) does not show the granulo reaction. The explanation appears in the results of the fermentation tests with the various carbohydrates (table 6). Only the significant differential carbohydrates are included.

My reactions of Wis 5, originally from Winogradsky, check with those of McCoy *et al* (1930), but deviate from Winogradsky's (1902) original description of *C. pasteurianum* regarding glycerol, mannitol, and dextrin. Such divergences may be due to methods or to strain variations, of which he observed several.

Strain Wis 60, a Lister Institute strain from Bredemann, is obviously not a true *C. pasteurianum* type, especially as shown by the fermentation of both lactose and cornstarch. It appears, therefore, to be merely one of the many ill-defined butyrics, probably close to the "*B. saccharobutyricus*" type of McCoy *et al* (1926). Strain Wis 68, originally from Kluyver, checks with the limited original description of *C. beijerinckii*, named by Donker (1926). This strain is obviously distinct from both Wis 5 and Wis 60.

#### SUMMARY

A method of testing for granulo in a semisolid glucose agar is described.

This reaction appears to set two butyrics (types or "species"), *Clostridium beijerinckii* and *Clostridium pasteurianum*, apart from all other members of the group included in this study.

This reaction seems to have a differential value equivalent to the "stormy fermentation" of *Clostridium perfringens*, the "iron-gelatin" reaction of *Clostridium histolyticum*, and the "vanillin-violet" (skatol) reaction of *Clostridium sporogenes*.

For the two species giving the positive granulo reaction the pH 6.6 limitation of Svartz does not apply. It does, however, appear to do so for all other butyrics included in this study.

These two strains formed granulo, in diminishing amounts, detectable in all glucose dilutions from 2.0 per cent through 0.05 per cent, inclusive.

That these reactions are fixed, and not evanescent, characters is proved by the fact that the same strains, separated in the laboratories of Dr. McCoy, Dr. Hall,

and the author for 7, 9, and even 11 years, have proved identical in every particular in duplicate and in triplicate cultures

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# THE EFFECTS OF *VIBRIO FETUS* ON THE GROWTH OF *TRICHOMONAS FOETUS* (PROTOZOA)<sup>1</sup>

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*Vibrio fetus* and *Trichomonas foetus* are pathogenic in the reproductive tract<sup>†</sup> of cattle and occasionally produce early abortion. Since the discovery by Smith (1918) of spirilla in cases of cattle abortion in the United States, instances of abortion resulting from vibronic infection have been sporadically reported in the literature (Barger, 1928, Plastringe *et al.* 1943, 1947, Rhoades and Hardenbrook, 1947).

The organisms are usually isolated from the rumen of aborted fetuses, amniotic and allantoic fluid, or fetal membranes. So far as known the two organisms have never been isolated simultaneously from the same animal.

Numerous reports in the literature indicate that many cases of bovine trichomoniasis are accompanied by a varied bacterial flora. The role or associative action of these organisms in the establishment of trichomonas infection is not known. The purpose of this paper is to present data concerning the effect of *V. fetus* upon the growth of *T. foetus in vitro*. This work is a continuation of earlier studies by Johansson *et al.* (1947).

## MATERIALS AND METHODS

**Culture medium.** A modification of Schneider's citrate medium was prepared and used in the same manner as by Johansson *et al.* (1947) with the exception that 0.3 per cent agar was added to the supernatant liquid. The final pH was 6.67.

In an attempt to develop a culture medium which would support the growth of both *V. fetus* and *T. foetus*, a quantity of the modified Schneider's medium was filtered with 10 per cent by weight of "norite" (activated carbon) just before the agar was added. The "norite" was added to the supernatant and brought to a boil. When the mixture reached the boiling point it was poured into a funnel containing a double layer of filter paper. The filtrate so obtained was water-clear, nor did it become turbid on sterilization as does the unfiltered medium.

When the vibrio was grown in this medium at 37°C in an atmosphere of 10 per cent carbon dioxide, according to the method of Plastringe and Williams (1943), a subsurface gray band from 12 to 18 mm wide was obtained. This is in contrast to the 2- to 4-mm band of growth obtained on the Plastringe and Williams' (1943) liver infusion medium containing 0.3 per cent agar, or on the unfiltered, modified Schneider's medium. The trichomonads failed to thrive in

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Project 622-V, Trichomoniasis and other reproductive diseases of cattle.

this medium, consequently, it was discarded. Mention is made of this medium because of the apparently greater amount of growth possible with *V fetus*, which conceivably could be of use in studies concerned with antigen production.

*Organisms employed* *Trichomonas foetus* used in this study was a strain isolated by Morgan and Wisnicky (1942) from a cow with a trichomonad pyometra and has since been maintained in pure culture on the modified Schneider's medium. *Vibrio fetus* was originally isolated from the rumen contents of a 5-month-old aborted fetus and was maintained on Plastring and Williams' (1943) medium until transferred to Schneider's modified medium.

*Determination of growth rates* The method of determining the growth rates was identical to that used by Johansson *et al* (1947). All cultures were incubated at 37 C. The trichomonad control was incubated in an atmosphere of 10 per

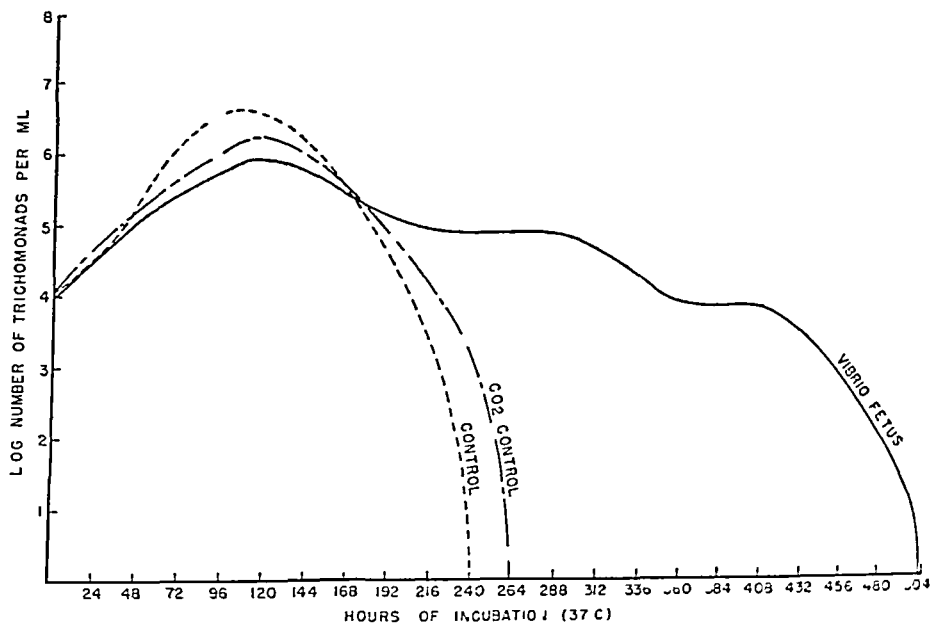


FIG 1 Growth of *TRICHOMONAS FOETUS* with *VIBRIO FOETUS*

cent carbon dioxide during its entire growth period. The vibrio plus the trichomonads, however, were incubated in an atmosphere of 10 per cent carbon dioxide for the first 3 days. After 3 days in carbon dioxide, the incubation was continued in atmospheric air to conform with Plastring and Williams' method of initiating the growth of the vibrio. It was believed that any effect that the addition of carbon dioxide might have on the extension of the growth of the trichomonads, after the first 3 days, could be detected in the carbon dioxide control. Johansson's atmospheric air control was used in this study.

To establish the growth curve, 50 replicates of tubes containing 5 ml of Schneider's medium and 50,000 trichomonads each were inoculated simultaneously with a 6-mm loopful of 3-day-old growth of *V fetus*. The pH of the vibrio-trichomonad cultures was checked with a Beckman potentiometer.

## RESULTS

The control cultures of trichomonads used were obtained by employing an inoculum of 50,000 organisms (trichomonads) in 5 ml of medium and subjecting the cells to an atmosphere of 10 per cent carbon dioxide throughout the entire incubation period. In this control there was no lag phase. The accelerated growth phase was similar to that of Johansson *et al* (1947), but the peak was attained in 120 hours, or 2½ hours later. The count at the time of the peak was approximately 1.3 million organisms per ml. The accelerated death phase and the logarithmic death phase were at 130 to 168 hours and 168 to 216 hours, respectively (figure 1). After 216 hours the numbers of trichomonads decreased rapidly, but not so rapidly as in Johansson's control.

The trichomonads in association with *V. fetus* were extended in their growth to 50½ hours, or 21 days. The peak of growth was reached at the end of 120 hours. After 120 hours the protozoa exhibited the accelerated death phase up to 216 hours, at which time they entered the stationary growth phase. The protozoa remained in this stationary growth phase to 288 hours, at which time they entered the accelerated death phase again at 360 hours. At the end of 360 hours the stationary phase again was maintained until 408 hours. After 408 hours the accelerated death phase was entered for the last time. At 432 hours the logarithmic death phase was reached, and the trichomonads finally became extinct at the end of 50½ hours.

It was noticed, also, that the change in pH or the formation of acid was slower in the vibrio-trichomonad mixed culture than in the control cultures. The pH in the control cultures was comparable to that found by Morgan (1942). In the vibrio-trichomonad culture a pH level of 4.9 was reached in 11 days. The pH did not go below 4.9 on succeeding days.

## DISCUSSION

The trichomonad controls in this study, with an atmosphere of 10 per cent carbon dioxide and in a medium containing 0.3 per cent agar, never reached the height in numbers that those in Johansson's controls did. Apparently the agar or the presence of the carbon dioxide had a restraining and extending effect on the development of the protozoa. Whether the trichomonad assimilated some carbon dioxide or the lowering of the oxygen tension caused the extension of trichomonad growth was not established. In view of the fact that the consistency of the medium has a definite effect on the growth of the vibrio, it would not seem unreasonable to assume that it might have some effect on the trichomonads.

Trichomonads growing in association with *V. fetus* apparently benefited from the stimulating substances liberated from the nutrient medium by the metabolic activities of the vibrio. Since the presence of *V. fetus* in the culture of the trichomonads retarded the change in pH during the first few days of growth, this also may be a factor in the prolongation of trichomonad activity.

## SUMMARY

*Vibrio fetus* extended the *in vitro* growth of *Trichomonas foetus*, as determined by direct microscopic counts of the trichomonads with a hemocytometer. An atmosphere of 10 per cent carbon dioxide extended the *in vitro* growth of trichomonads by 24 hours over the normal control.

These extensions are believed to be caused by (1) the release of nutrients favorable to the growth of the trichomonads, (2) the slower rate of change in the hydrogen ion concentration of the culture medium, and (3) the lowering of the oxygen tension of the atmosphere with carbon dioxide.

Filtration of Schneider's modified medium with "norite" increased the area of growth of *Vibrio fetus*.

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# THE INFLUENCE OF VARIED PROTEIN INTAKE AND OF TRYPTOPHANE DEFICIENCY ON THEILER'S ENCEPHALOMYELITIS OF MICE<sup>1</sup>

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From a series of studies on the effect of nutrition on poliomyelitis in various animals, we have reported some of the results obtained with vitamin deficiencies, inanition, and mineral deficiencies. Of the vitamins studied, thiamine has been found to have the greatest influence, since a deficiency of this vitamin resulted in decreased susceptibility of mice to the Lansing strain of poliomyelitis virus, as evidenced by fewer paralyses and a lower death rate. Restriction of the total food intake or simple caloric restriction did not reproduce these results, although both the development of paralyses and death were delayed (Rasmussen *et al*, 1944a). Similar observations have been reported by the Philadelphia group (Foster *et al*, 1944). Thiamine deficiency also resulted in a marked reduction in infections with Theiler's GDVII (Waisman *et al*, 1945) and Theiler's TO (unpublished results) viruses. With the latter virus the results are less clear, since the incubation period may be long, and no attempt was made to maintain the deficient mice beyond the period when deaths due to deficiency appeared. With Theiler's FA virus the results were equivocal (Rasmussen *et al*, 1944a). Restrictions of carbohydrate or of the diet as a whole with GDVII virus resulted in clear evidence of infection in about 50 per cent of the animals, and death in the rest, without paralysis or encephalitic signs, although many of the mice manifested early signs of the disease before dying. Deaths were not delayed and the incidence was not reduced. Restriction of fat content had no effect (unpublished results). A deficiency of pantothenic acid in mice produced no change in resistance to the Lansing strain but resulted in decreased susceptibility to Theiler's GDVII virus (Lichstein *et al*, 1944). Riboflavin deficiency, on the other hand, had a slight effect in increasing resistance to the Lansing virus, but no effect against the Theiler's GDVII or FA viruses (Rasmussen *et al*, 1944b). Pyridoxine, inositol, and biotin deficiencies were found to be inactive against infection with the Lansing and GDVII viruses (Lichstein *et al*, 1945). Para-aminobenzoic acid, which has striking antirickettsial activity, had no influence on infection with the GDVII virus at concentrations in the diet ranging from none to 10 per cent (unpublished results).

In the studies with minerals, deficiencies of calcium, magnesium, chlorine, and sodium (Lichstein *et al*, 1946b) and a high intake of fluorine (unpublished results)

<sup>1</sup> These studies were aided in part by a grant from the National Foundation for Infantile Paralysis, Inc., New York.

had no effect, whereas deficiencies of potassium or phosphorus resulted in a greatly reduced incidence of paralysis with Theiler's GDVII virus (Lichstein *et al*, 1946b). In the last two deficiencies the number of paralyzed animals was increased as the amounts of phosphorus or potassium in the diets were increased. It should be noted, however, that although the incidence of paralysis varied, deaths without signs of infection invariably brought the total fatality rate in these mice to 100 per cent.

Attempts to improve the resistance of mice by improving the diet have not been successful to date. The addition of liver preparations to the mouse diet and higher fat content (15 or 25 per cent corn oil or butter fat) produced no change in resistance to the GDVII virus, although excellent weight gains were obtained on some of the rations (unpublished results).

In poliomyelitis of monkeys vitamin deficiencies have been relatively ineffective. Deficiencies of thiamine (Clark *et al*, 1945), biotin, and ascorbic acid (unpublished results) produced no change in susceptibility. Folic acid deficiency when acute had no effect, but when chronic a reduced incidence of poliomyelitis resulted (Lichstein *et al*, 1946a). Potassium deficiency, likewise, has no effect on poliomyelitis in the monkey (unpublished results). When monkeys were fed our so-called "super" diet, in which extra vitamins, whole liver substance, plasma globulin, and yeast were included, the results with poliomyelitis were suggestive of increased resistance in one experiment, but this could not be repeated (unpublished results).

Some of these studies have been extended to other viruses—avian encephalomyelitis in chicks and Western equine encephalomyelitis in mice. The incidence of avian encephalomyelitis depended on the age of the chick, the previous state of nutrition, and the amount of thiamine in the diet (Cooperman *et al*, 1946). When the chicks were inoculated at the age of 1 day, those receiving an optimum level of thiamine were protected to a greater extent than those receiving low or suboptimum levels. When the chicks were inoculated after receiving an optimum diet for 2 weeks, thereafter receiving the experimental diets, those receiving the lowest level of thiamine were protected to the greatest degree.

Thiamine deficiency in animals infected with Western equine encephalomyelitis virus resulted in a marked modification of the course of infection, since most of the mice died without showing typical signs of infection. Titrations of the brains of these mice showed, however, that the virus had multiplied in these animals as well as in the mice on an optimum diet (Kearney *et al*, in manuscript). Inanition and potassium deficiency (unpublished results) had no effect in this virus disease.

In view of the importance of amino acids in the building of proteins, be it virus or host, we have been studying deficiencies of protein and of amino acids. Although preliminary experiments with lysine, valine, and methionine deficiencies are suggestive of interesting consequences in infection with the GDVII virus, only the work with protein and tryptophane deficiencies is complete enough to report at this time. Jones *et al* (1946) have recently reported the effect of low protein and low tryptophane diets in mice with the Lansing strain of poliomyelitis.

virus They found that low protein diets (5 per cent casein) produced a slight delay and the low tryptophane diet a pronounced delay in the onset of poliomyelitis Paralysis was, however, observed in all mice before death

#### MATERIALS AND METHODS

*Mice* Swiss mice raised in our laboratory were used in all of these experiments<sup>2</sup> Mice were placed on the experimental diets when they were from 20 to 26 days old, and a split litter technique with consideration for weight and sex of the individual animals was employed throughout The mice were kept, as described previously (Rasmussen *et al.*, 1944a), individually in screen-bottom cages with food and water available at all times

*Diets* The composition of the various diets used in these experiments is given in table 1 Diet no 1 is our regular synthetic optimum diet Mice receiving this ration appeared sleek and healthy and gained an average of 4 to 5 g during the first week, and continued to gain throughout the experimental period, the average weight gain for 1 month being about 10 g Diet no 2 was the same except for the casein content, which was raised to 36 per cent When dietary components were increased or decreased, the amount of sucrose was either decreased or increased accordingly Mice receiving the 36 per cent casein diet gained at a faster rate than those on the 18 per cent casein diet, but reached a peak of about 9 g gain in 2 weeks, after which they leveled off On diet no 3, containing 15 per cent casein, the average weight gain was 3.5 g the first week, the mice continued to gain throughout the experimental period and averaged a total weight gain of 9 g in 3 1/2 weeks Mice fed diet no 4, 9 per cent casein, gained 2 to 3 g the first week and 7 to 9 g during a 3- to 4-week period Growth was not so good on this ration as on those with higher levels of casein, but the mice appeared healthy Since the amount of cystine is low when casein is fed at the 9 per cent level, this diet was supplemented in some of the rations with cystine Rats fed 9 per cent casein diets plus 6 per cent gelatin show a retardation of growth, which can be overcome either by the addition of tryptophane or niacin, these factors were included, therefore, in some of the diets Mice on these rations (diets 5 to 8) gained at about the same rate as those on the unsupplemented 9 per cent casein diet and appeared healthy Diet no 9 contained the ten essential amino acids in pure form, but no protein, the weight difference in the diet being made up by extra sucrose The amino acids were present as 10.9 per cent of the diet, or 8.4 per cent as active isomers<sup>3</sup> Sodium acid phosphate is included in the amino acid diets to replace the phosphorus present in 18 per cent casein On this diet mice maintained their weights or lost 0.5 to 1.0 g during the first 4 or 5 days, after which small weight gains brought the weights up to the original values During a 3- or 4-week period weights were maintained within 1 g with occasional gains of 2 g above the original weights Diet no 10 is the same diet as no 9 except for 2 per cent casein, which was added to supply the peptide linkage Response to this diet was similar to that described

<sup>2</sup> Original source was the laboratory of Dr Webster

<sup>3</sup> Abbreviated from now on to 8.4 per cent EAA diet

for diet no 9 Diet no 11 consisted of the same ingredients as no 9, except that tryptophane was absent Mice fed this ration lost an average of 2.0 to 2.5 g

TABLE 1  
Composition of diets

INGREDIENTS	DIET 1	DIET 2	DIET 3	DIETS* 4 5	DIET 6	DIET 7	DIET 8	DIETS* 9 10	DIETS* 11, 12	DIET 13	DIET 14	DIET 15
Sucrose	73.2	51.2	75.2	81.2 (81.0)	75.2	75.2	74.9	78.67 (76.67)	78.97 (76.97)	78.17	74.2	73.9
Casein	18.0	36.0	15.0	9.0	9.0	9.0	9.0	— (2.0)	— (2.0)	—	—	—
Gelatin	—	—	—	—	6.0	6.0	6.0	—	—	—	—	—
Salts IV†	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mixture‡	0.5	0.5	0.5	0.5	—	0.5	—	0.5	0.5	1.0	1.0	1.0
Niacin-deficient vitamin mixture§	—	—	—	—	0.5	—	0.5	—	—	—	—	—
Choline	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.6	0.6	0.6
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cystine	—	—	—	—	—	—	—	—	—	—	0.2	0.2
Tryptophane	—	—	—	(0.2)	—	—	0.3	—	—	—	—	0.3
Amino acid mixture	—	—	—	—	—	—	—	10.9	10.6	10.6	—	—
Acid-hydrolyzed casein	—	—	—	—	—	—	—	—	—	—	15.0	15.0
NaH <sub>2</sub> PO <sub>4</sub>	—	—	—	—	—	—	—	0.63	0.63	0.63	—	—

No 1 = 18% casein regular optimum diet, no 2 = 36% casein diet, no 3 = 15% casein diet, no 4 = 9% casein diet, no 5 = 9% casein diet + cystine, no 6 = 9% casein + 6% gelatin, niacin deficient, no 7 = 9% casein + 6% gelatin, niacin present, no 8 = 9% casein + 6% gelatin, niacin deficient, tryptophane supplemented, no 9 = 8.4% essential amino acid (EAA) diet, no 10 = 8.4% EAA diet + 2% casein, no 11 = 8.4% EAA diet minus tryptophane, no 12 = 8.4% EAA diet minus tryptophane + 2% casein, no 13 = 8.4% EAA diet minus tryptophane + double portions of vitamins, no 14 = acid-hydrolyzed casein diet + double vitamins + cystine, tryptophane deficient, no 15 = acid hydrolyzed casein diet + double vitamins + cystine + tryptophane

\* Diets 4 and 5 differ only in cystine and sucrose content The numbers in parentheses refer to no 5 Diets 9 and 11 differ from 10 and 12, respectively, only in casein and sucrose content The numbers in parentheses refer to nos 10 and 12 in the respective columns

† Phillips and Hart salts (Phillips and Hart, 1935)

‡ Added as a dry mixture containing the following parts per 100 g of diet: thiamine 300 µg, riboflavin 300 µg, pyridoxine 300 µg, niacin 500 µg, Ca-pantothenate 20 mg, L-inositol 100 mg, *p*-amino benzoic acid 100 mg, biotin 10 µg, folic acid 25 µg In addition, adequate amounts (2 drops) of oleum percomorphum were fed by mouth each week

§ Contained no niacin, otherwise the same as that given above

|| Amino acid mixture contains the following parts per 100 g of diet: L-(+) lysine HCl H<sub>2</sub>O 1.5, *DL*-tryptophane 0.3, L-(+)-histidine HCl H<sub>2</sub>O 0.6, *DL*-phenylalanine 1.0, L-(−) leucine 1.2, *DL*-isoleucine 1.5, *DL*-threonine 1.5, *DL*-methionine 0.9, *DL*-valine 2.1, L-(+) arginine HCl 0.3 In the tryptophane-deficient diets (nos 11, 12, 13) this mixture contained no tryptophane Amino acids were purchased from Merck and Company

the first week, leveled off in weight somewhat the second week, and then lost weight gradually, the total weight loss during a 4-week period being about 1 g

When 2 per cent casein was added (diet no 12), the weight losses were similar. Diet no 13 contained double portions of all vitamins, but other than this was identical with no 11. In diet no 14, acid-hydrolyzed casein at 15 per cent of the ration was used as the nitrogen source. At this level the ration is low in cystine, this amino acid was therefore added to the ration in 0.2 per cent concentration, and tryptophane, which was destroyed during the hydrolysis, was left out, making this diet tryptophane-deficient, but supplying the other essential and the nonessential amino acids. Double portions of vitamins were also given in this ration. Weight losses occurred of the same order of magnitude as with the other tryptophane-deficient diets. In diet no 15 the no 14 ration was completed by the addition of tryptophane at 0.3 per cent. Mice fed this diet lost about 0.5 g during the first week and then gained about 2 g during the second week. The initial weight loss was slightly less and the following gain more than in mice fed the 8.4 per cent EAA ration (diet no 9).

Mice maintained on the tryptophane-deficient rations, diets no 11 to 14, showed the following signs of deficiency. Loss of weight began immediately and continued at a slower rate after the first week. Slight irritability, weakness—especially of the hind legs—and tremors were among the early signs, these became more apparent in about 2 weeks. The hind legs were spread to the side, almost at right angles to the body (abduction), and the hip region became low, simulating in appearance a pelvic paralysis, although the muscles were merely weak, not paralyzed. Such signs were more noticeable after the mice were twirled by the tail and dropped on the table, at times the mice would react violently to such treatment—hopping and jumping about with the head thrown back as in opisthotonus, or they would appear to “scramble” along in marked ataxia, with all legs moving rapidly, but only the front legs at all effective in propelling the mouse. Tremors and some revolving were evident when the animals were held by the tail and spun. As the deficiency became more severe, all of these signs became more marked. The mice sat with their heads in the food cups, their backs were humped with both the head and hips close to the table, and they appeared to be mainly head and shoulders. Tonic convulsions with the hind legs completely extended and the fore limbs flexed were frequently observed. During such convulsions the mice became rigid and cyanotic and appeared dead. Death sometimes occurred during these seizures, but more often the mouse would gradually relax after a few seconds and commence breathing. At times the convulsions seemed incomplete in the sense that the hind legs and lower region of the body were stiff and extended but the front legs were moving convulsively. Rapid movements of all legs were often observed just before the extension and flexion of the convulsion. Tonic convulsions were seen more readily after vigorous spinning but also occurred spontaneously. Many of the mice would undergo such convulsions 4 and 5 times during a period of a few days before death supervened. Weakness increased to the point of prostration in the deficient mice, and death occurred after from 18 to 35, or more, days on the diet.

Mice receiving tryptophane evidenced no signs such as these except for a

tendency to spread the hind legs in one or two mice that did not respond well to the 8 4 per cent EAA diet

*Virus* The virus used in these experiments and in many of our previous series was received by us as Theiler's GDVII strain of mouse encephalomyelitis virus from the Rockefeller Institute in 1940 and has since been maintained in this laboratory by intracerebral passage in mice. In our hands, however, this virus has not produced in mice the disease described by Olitsky (1915) as characteristic for the GDVII virus. In view of the recent criticism of the use of the GDVII virus as a model for poliomyelitis (Olitsky, 1945, Schneider, 1946) we feel that further clarification is needed of the type of infection produced by the virus with which we have been working. The first signs of infection following intracerebral inoculation of this virus into mice occur in about 5 to 10 days, the average being 7 to 8 days. The onset of the disease is characterized by hyperirritability, circling, spasmodic and convulsive movements, and, occasionally, tonic-clonic convulsions. Encephalitic signs are more marked in young mice (21 to 28 days old) and less marked in older animals (35 to 40 days old). These signs are rarely so severe as in an infection such as Western equine encephalomyelitis in mice and may not be seen without the stimulation of spinning by the tail. The encephalitic stage may last for 3 or 4 days but usually is followed in 24 hours by a poliomyelitic stage. Cord involvement is apparent first by a tendency to save or favor one leg, and this progresses through partial to complete flaccid paralysis of the limb. The paralysis then progresses during the course of another day to involvement of other muscle groups, resulting at times in quadriplegia, shoulder, hip, back, and side paralyses. Death soon follows severe paralysis. The mice may die at any stage of the infection, but more often the disease runs its characteristic course for 3 or 4 days from the onset of symptoms to death. Definite flaccid paralysis is seen in almost 100 per cent of the mice, and death has been invariable. On intraperitoneal injection paralysis follows in 9 to 10 days and encephalitic signs are less noticeable.

The signs of this disease are in contrast to those described by Olitsky (1915) for the GDVII virus, who states that there is only occasional weakness or paralysis of one or more limbs and that signs invariably precede death by only a few hours. It is in general agreement, however, with the early description by Theiler and Gard (1940), who described the cardinal symptoms of this infection as a flaccid paralysis of the limbs. The behavior of Theiler's TO virus in our mice is similar to that described both by Theiler (1937) and by Olitsky (1915). There can be no doubt that the virus we have been using is not TO, but whether it is the GDVII strain or a spontaneous virus remains to be decided.

In the experiments reported here the source of virus was infected mouse brains preserved in 50 per cent glycerol-saline solution. The mouse brains were washed with normal saline, ground with alundum, and suspended in normal saline at 10 per cent concentration, the 10 per cent suspension was allowed to settle or was centrifuged lightly and the supernatant diluted with normal saline to 1 0 per cent or 0 1 per cent of the original weight of brains. Mice were routinely injected intracerebrally under light ether anesthesia with 0 03 ml of the diluted suspension. When normal brain material was injected, the source was normal.

mouse brains preserved in the 50 per cent glycerol-saline solution. These were prepared in the same manner and injected in the same concentration as the virus-containing brains in a given experiment. The mice were observed twice daily for signs of infection or deficiency, beginning 4 days after inoculation, for a 28-day period, at which time the experiments were usually terminated. During the observation the mice were spun vigorously for a few seconds while held by the tail to aid in eliciting encephalitic signs and were allowed to walk about and down the cage to test for paralysis and strength of grip.

Mice dying within 3 days after inoculation were excluded from the final tabulations to eliminate those which may have died from trauma or those which did not respond to the synthetic diets.

#### EXPERIMENTAL PROCEDURES AND RESULTS

*Low protein diets* Prior to the series here reported we had conducted a number of experiments on the influence of the kind and level of protein in the diet on infection with Theiler's GDVII virus. In these experiments mice were fed diets containing casein at 9, 13, 15, 18, and 21 per cent levels. In some cases cystine, niacin, and tryptophane supplements were made, and in others the protein was supplemented with gelatin or zein. The results in all cases in which the level of protein was 15 per cent or less were the same.

The incidence of paralysis was low compared to that in mice receiving 18 or 21 per cent casein, but most of the animals died before paralysis or encephalitic signs were observed. Control mice that were not inoculated or were inoculated with normal brain suspensions did not die but showed poor growth. Since other workers in our laboratories had obtained good growth in mice fed diets containing 13 or 15 per cent casein, we suspected the adequacy of our basal ration. When these studies were initiated, the vitamins were added to the ration by dissolving them in an alcohol solution, adding the solution to a given amount of casein, drying, and using measured amounts of this fortified casein in the final ration. When the vitamins were added as a dry mixture, better growth was obtained, and when a mixture of the vitamins was given to the mice showing growth failure, significant growth responses occurred. Similar results were obtained when thiamine alone was added, indicating a destruction of this vitamin during the preparation of the fortified casein. Since we had used similar procedures without difficulty in other work, it appears that the casein must have contained small amounts of sulfite, which is highly destructive to thiamine. Inasmuch as neither the deficiency signs nor the response of these mice to the virus was characteristic of thiamine deficiency, other nutritional complications may have arisen from a combination of the low protein diet and the anorexia associated with the vitamin deficiency.

These results have not been reported in detail since they are not based on diets low only in protein, they are mentioned only because they demonstrate the difficulties that may be encountered in nutrition studies, in spite of utmost care in preparing rations, and because they offer still another example of the influence nutrition may have on a virus infection.

The low protein diets were then restudied, with the dry vitamin mixture used,

in three experiments, series 81, 84, and 87. In series 81, 140 mice, 23 days old, were divided into eight groups. Group 1, 7 mice, received 36 per cent casein (diet no. 2) and were not inoculated. Group 2, 28 mice, received the same diet and virus inoculation. Groups 3 and 4, with 7 and 28 mice, respectively, received the 18 per cent casein diet (diet no. 1), group 3 remaining uninoculated and group 4 receiving the virus. Groups 5 and 6 were fed the 9 per cent casein diet (diet no. 4), group 5, with 7 mice, remaining uninoculated and group 6, 28 mice, being inoculated with virus. Groups 7 and 8 received the 9 per cent casein diet with a supplement of cystine (diet no. 5). Group 7, with 7 mice, was the uninoculated control group, and group 8, 28 mice, received virus inoculation. The

TABLE 2  
*Effect of low protein diets on incidence of virus infection*

DAYS AFTER INOCULATION	INCIDENCE OF VIRUS INFECTION EXPRESSED IN CUMULATIVE PERCENTAGES OF TOTAL MICE IN EACH GROUP													
	Series 81				Series 84								Series 87	
	Group 2 (28)* Diet 2	Group 4 (28) Diet 1	Group 6 (27) Diet 4	Group 8 (27) Diet 5	Group 1 (6) Diet 1	Group 3 (20) Diet 3	Group 5 (24) Diet 4	Group 7 (26) Diet 5	Group 9 (22) Diet 7	Group 11 (21) Diet 6	Group 13 (24) Diet 8	Group 2 (33) Diet 4	Group 4 (33) Diet 5	Group 6 (37) Diet 1
6	0	7	4	0	33	5	4	4	19	19	8	0	0	3
8	64	68	41	41	50	25	46	43	54	18	62	21	9	6
10	100	96	55	89	100	70	83	92	82	95	92	51	36	31
12	100	100	63	96	100	85	92	100	95	100	92	91	73	72
14	100	100	74	96	100	100	100	100	95	100	92	97	82	87
16	100	100	74	100	100	100	100	100	95	100	92	97	88	91
18	100	100	74	100	100	100	100	100	95	100	92	100	88	91
Total														
% pvi†	100	100	74	100	100	100	100	100	95	100	92	100	88	91
% par‡	93	96	67	89	100	95	96	100	77	95	88	85	79	75
% f§	7	4	4	0	0	5	4	0	18	5	4	6	0	0

Days = day when definite paralysis occurred, or day of death if no paralysis

\* Number of mice surviving through third day after inoculation

† pvi = positive virus infection, i.e., typical signs of infection, includes paralysis

‡ par = paralysis, included in table as pvi

§ f = death without typical signs of infection, included in the table as pvi

groups were inoculated after 11 days on the rations with a 10 per cent suspension of the virus. The results of this series are summarized in table 2. In all groups the majority of the inoculated mice showed definite paralysis. In group 6 the incidence of infection is 26 per cent less than in the other groups, the rest of the mice surviving. In each of groups 2, 4, and 6, one mouse died without showing signs of infection. In group 8 frank paralysis occurred in 89 per cent of the mice, with the remaining 11 per cent (3 mice) dying after signs of encephalitis but before paralysis was observed. No deaths occurred in the control groups.

In series 84 a total of 210 mice, 20 to 23 days old, were placed on the following rations: group 1, 18 per cent casein (diet no. 1), groups 2 and 3, 15 per cent casein (diet no. 3), groups 4 and 5, 9 per cent casein (diet no. 1), groups 6 and 7,



9 per cent casein with cystine supplement (diet no 5), groups 8 and 9, 9 per cent casein plus 6 per cent gelatin, with niacin supplement (diet no 7), groups 10 and 11, 9 per cent casein, 6 per cent gelatin, no supplements (diet no 6), groups 12 and 13, 9 per cent casein, 6 per cent gelatin, with tryptophane supplement (diet no 8). The even-numbered groups were the uninoculated control groups with 7 mice in each group. The odd-numbered groups were all inoculated with a 10 per cent suspension of the virus after 13 days on the diets. Group 1 contained 7 mice, group 3, 21 mice, and the others contained 28 mice each. The results, presented in table 2, show no significant differences in any of the groups. In each of groups 3, 5, 11, and 13 one died without signs of infection. In group 9, 4 mice died in this manner, making the totals in this group 77 per cent paralyzed, 18 per cent died without signs of infection, or 95 per cent total deaths.

In series 87 the 9 per cent casein diets were again studied. A total of 126 mice, 23 to 26 days old, were divided into groups as follows: groups 1 and 2 received the 9 per cent casein ration (diet no 4), groups 3 and 4 received this diet supplemented with cystine (diet no 5), and groups 5 and 6 the 18 per cent casein diet (diet no 1). Groups 1, 3, and 5, consisting of 7 mice each, were the uninoculated controls. Groups 2, 4, and 6, with 35 mice each, were inoculated with virus. These groups were injected after 11 days on the rations with a 0.1 per cent suspension. The results, given in table 2, show an incidence of infection essentially the same regardless of the diet.

The findings of the low protein experiments indicate that reduction of the protein intake of mice to 9 per cent of the diet, when the protein source is casein, has no influence on infection with Theiler's GDVII virus.

*Tryptophane-deficient diets.* Three experiments were conducted on the effect of tryptophane deficiency on infection with Theiler's GDVII virus. The first of these, series 88, was a preliminary experiment with various amino acid deficiencies, but only the results with tryptophane will be here presented. Four groups of 14 mice, 24 to 26 days old, were placed on the following diets: group 1, tryptophane-deficient (diet no 11), group 2, tryptophane-deficient plus 2 per cent casein to supply the peptide linkage, or "strepogenin" factor (diet no 12), group 3, the ten essential amino acids supplied in pure form, but no nonessential amino acids, the final concentration of the active isomers being 8.4 per cent of the diet (diet no 9), group 4, the same as in group 3 but with 2 per cent casein added (diet no 10). All groups were inoculated after 5 days on the rations with a 0.1 per cent suspension of the virus. The results, summarized in table 3, even with such small numbers of animals were suggestive enough to warrant further study. In group 1 none of the mice showed evidence of infection, the only signs seen being those referable to the deficiency, such as hind leg weakness, a tendency to spread the hind legs, tremors, and tonic convulsions. In group 2, three mice gave evidence of virus infection. Two of these showed only an indication of encephalitis—a tendency to paw the air and face spasmodically, with death on the twelfth and fourteenth days after inoculation. The third mouse appeared to have a partial paralysis of the right front leg on the twenty-sixth day. For simplicity these are included in the table as fatalities. The two groups

receiving the full complement of the essential amino acids responded well to the virus, showing signs of the disease in its characteristic course in most cases, when paralysis was not observed, definite encephalitic signs were evident. The final death rates in all four groups were approximately the same.

The second experiment, series 93, was a more effective study of tryptophane deficiency. A total of 147 mice, 21 to 23 days old, were placed on the rations as follows: group 1, 14 mice, received the 8.4 per cent EAA diet (diet no. 9), group 2, 14 mice, received the same diet and were inoculated with a 0.1 per cent suspension of normal brains. Group 3, 28 mice, received the same diet and were inoculated with a 0.1 per cent suspension of virus. Group 4, 14 mice, com-

TABLE 3  
*Results of series 88*

DAYS AFTER INOCULATION	GROUP 1 (12),* DIET NO 11 TRYPTOPHANE DE FICIENT	GROUP 2 (13) DIET NO 12 TRYPTOPHANE DE FICIENT + 2% CASEIN	GROUP 3 (11) DIET NO 9, 8.4% EAA	GROUP 4 (14), DIET NO 10, 8.4% EAA + 2% CASEIN
	% f	% f	% p†	% p†
11	17	15	18	14
13	42	38	45	36
15	42	54	55	43
17	42	54	55	57
19	42	54	73	57
21	42	62	73	64
27	58	69	73	64
Total				
% pvi†	0	23	73	57
% par‡	0	0	55	36
% f§	58	46	0	7
% F	58	69	73	64

\* Numbers in parentheses = numbers of mice surviving through the third day after inoculation.

† pvi = positive virus infection, i.e., typical signs of infection, includes paralysis.

‡ par = paralysis, included in table as pvi.

§ f = death without typical signs of infection.

|| F = total fatality regardless of signs shown.

pared the uninoculated tryptophane-deficient control group (diet no. 11). Group 5, 21 mice, received the tryptophane-deficient diet and an injection of normal brain suspension. Group 6, 28 mice, received the tryptophane-deficient diet and were inoculated with virus. Group 7 consisted of 28 mice, 14 of which received the 18 per cent casein optimum diet (diet no. 1) and 14 this diet minus the corn oil; these are included together as one group since no differences were found between them either in response to the diets or to the virus. Inoculations of normal brain suspension and virus were performed after 7 days on the diets. The results of this experiment are presented in table 4. All mice in group 7 exhibited positive signs of infection, and only one mouse failed to show paralysis. These results are characteristic of the

many times we have used mice on the optimum ration and this virus. The group receiving tryptophane as part of the 8 1 per cent EAA diet (group 3) also showed a high percentage of virus infection, with unequivocal encephalitic signs and paralysis. The incidence of paralysis, however, was lower than in the optimum group, with 17 of the 25 mice (68 per cent) showing paralysis and 7 (28 per cent) dying before paralysis was observed. No deaths occurred in the control groups on this diet.

Mice receiving no tryptophane presented a markedly contrasting picture. Only one mouse of the 24 in this group (group 6) showed signs of infection and it developed a partial paralysis of the left front leg after a history of encephalitis. The rest of the animals in this group manifested only signs of deficiency—weakness, tremors, a tendency to spread the hind legs, especially after the mouse was

TABLE 4  
*Incidence of infection in series 93*

DAYS AFTER INOCULATION	GROUP 3 (25) DIET NO 9 8 4% EAA + VIRUS	GROUP 4 (12) DIET NO 11 TRYPTO- PHANE DEFICIENT, UNINOCULATED	GROUP 5 (19) DIET NO 11 TRYPTO- PHANE DEFICIENT + NORMAL BRAIN	GROUP 6 (24) DIET NO 11 TRYPTO- PHANE DEFICIENT + VIRUS	GROUP 7 (25) DIET NO 1 18% CASEIN OPTIMUM + VIRUS
	% pts	% F	% F	% F	% pts
7	12	0	0	0	16
9	32	8	0	4	60
11	60	8	0	13	88
13	80	8	0	46	96
15	88	8	11	58	100
17	96	17	21	71	100
19	96	17	21	83	100
21	96	50	26	83	100
29	96	67	58	96	100
Total					
% pvi	96	—	—	4	100
% par	64	—	—	4	96
% f	0	—	—	92	0
% F	96	67	58	96	100

See table 3 for explanation of symbols

spun by the tail, hunched posture, and tonic convulsions. The hind legs often appeared to be stiff, and in most cases quite weak, but the mice were able to walk, grip the cage, and walk down the cage, and were therefore considered not paralyzed. These signs of deficiency appeared earlier in many of the deficient mice receiving virus than in the two groups of controls and progressed to severe deficiency about a week earlier, considering the groups as a whole. The controls presented the same signs as the deficiency progressed. Death also occurred earlier in the virus-inoculated deficient mice than in the controls (table 4), reaching 71 per cent on the twenty-fourth day on the diet, or 17 days after inoculation, at which time the deaths totaled 17 per cent and 21 per cent in the uninoculated and normal brain-inoculated control groups. This suggests that these mice had died from virus infection, although no clinical signs were evident.

After 24 days on the diet, deaths in the control groups occurred more frequently, reaching 67 per cent and 58 per cent by the thirty-sixth day, it is therefore possible that the deaths occurring in the virus-inoculated group after 24 days (17 days after inoculation) may have been due to deficiency. If the deaths up to this time are considered to be due to virus infection, and the death rate of the deficient mice is compared to the two groups receiving tryptophane, there are still real differences in incidence up to the seventeenth day after inoculation, the percentages at this time totaling 96 in the group receiving the 8.4 per cent EAA diet, 100 per cent in the group receiving the 18 per cent casein, and 71 per cent in the tryptophane-deficient mice. From the time of inoculation until this time the delay of death in the deficient animals is consistent. Tonic convulsions were recognized in this series as a part of the deficiency syndrome, since mice in both control groups manifested this sign in a manner identical with the virus inoculated groups. In summary of this experiment then, it appears that tryptophane deficiency hindered and possibly prevented virus infection in many of the mice. Further, this deficiency radically altered the course of the disease, only one of the 24 mice showing signs of infection. Since many of these animals developed severe signs of deficiency and died earlier than the control deficient mice, it is possible that the virus infection increased the severity of the deficiency.

The third experiment, series 97, was conducted in repetition and extension of the previous experiments. The mice were 19 to 24 days old when placed on the rations, and many of the younger ones died. The number of mice given for each group is therefore the number surviving until 3 days after inoculation. Groups 1, 2, and 3 were fed the tryptophane-deficient diet as such (diet no. 11), group 1, 12 mice, remaining uninoculated, group 2, 15 mice, being inoculated with a suspension of normal brains, and group 3, 26 mice, being inoculated with a suspension of the virus. Groups 4 and 5 were fed the tryptophane-deficient diet, but with double portions of vitamins (diet no. 13) in an attempt to rule out vitamin-deficiency complications that might have arisen from the pronounced inanition associated with tryptophane deficiency. Group 4, 15 mice, was injected with normal brain suspension, and group 5, 26 mice, with the virus. In groups 6 and 7 a deficiency of tryptophane was produced by feeding as the nitrogen source acid-hydrolyzed casein, supplemented with extra cystine and double portions of vitamins (diet no. 14). It was hoped that this diet would eliminate complications due to the lack of nonessential amino acids and total nitrogen, as well as vitamins. Group 6, 13 mice, received an inoculation of normal brain material and group 7, with 17 mice, the virus. Group 8, 19 mice, received the same diet as groups 6 and 7 but with tryptophane added (diet no. 15). Group 9, 13 mice, received the 8.4 per cent EAA diet (diet no. 9) and group 10, 18 mice, the 18 per cent casein optimum diet (diet no. 1). The last three groups were all injected with virus. Both the normal brain and virus suspensions were inoculated in 0.1 per cent concentration after 8 days on the diets. Beginning on the fourth day after inoculation, the mice were thoroughly examined every hour from 8:00 A.M. until 10:00 P.M. daily for signs of infection and deficiency. These examinations were made of control animals as well as of virus-

inoculated groups each time. Following death, the brains and spinal cords, including the cervical region, from mice in all groups were removed and placed in 10 per cent formalin or 50 per cent glycerol-saline solution for histological study or titration of virus content. The results of this experiment are presented in table 5. With three different tryptophane-deficient diets, the results were the same. In each case most of the mice evidenced only signs of deficiency and none referable to the virus. In group 3 only 6 of the 26 mice (23 per cent) showed signs of infection. In 5 of these, encephalitis of a mild sort was the only sign, in the sixth, there was no encephalitis but a questionable hind leg paralysis on the thirteenth day after inoculation. In group 5 the extra vitamins had no apparent effect, since the deficiency signs and the response to the virus were

TABLE 5  
*Incidence of infection in series 97*

DAYS AFTER INOCULATION	GROUP 3 (26), DIET NO 11, TRYPTOPHANE DEFICIENT		GROUP 5 (26), DIET NO 13, TRYPTOPHANE DEFICIENT + DOUBLE VITAMINS		GROUP 7 (17), DIET NO 14, ACID HYDROLYZED CA SEIN		GROUP 8 (19), DIET NO 15, ACID-HYDRO- LYZED CASEIN + TRYPTOPHANE	GROUP 9 (13), DIET NO 9 8 4% EAA	GROUP 10 (18), DIET NO 1 18% CASEIN OPTIMUM
	% f	% pvi	% f	% pvi	% f	% pvi	% pvi	% pvi	% pvi
5	4	0	4	0	6	0	0	8	6
7	23	0	19	0	35	0	26	16	50
9	35	4	35	8	47	0	84	92	78
11	50	12	42	12	59	6	100	92	100
13	62	19	58	23	76	12	100	100	100
15	62	23	58	31	76	18	100	100	100
17	69	23	62	35	76	18	100	100	100
19	69	23	62	35	82	18	100	100	100
21	69	23	65	35	82	18	100	100	100
23	73	23	65	35	82	18	100	100	100
27	77	23	65	35	82	18	100	100	100
Total									
% pvi		23		35		18	100	92	100
% par		4		15		18	84	77	100
% f		77		65		82	0	8	0
% F		100		100		100	100	100	100

See table 3 for explanations of symbols

about the same as in group 3. There were four cases of paralysis in this group, but the total positive virus infection is again low. Mice receiving the acid-hydrolyzed casein diet (group 7) appeared similar in every way to those in group 3, and no effect could be seen from the extra nitrogen present in this diet. Of the 17 mice in this group, only 3 showed signs of infection, and each of these showed paralysis of the left front leg but no encephalitis. On this same diet plus tryptophane (group 8), 84 per cent of the mice became paralyzed and 100 per cent showed clear-cut signs of infection. In group 9, which received the same diet as group 3 but with tryptophane, 77 per cent of the mice became paralyzed, and all but one mouse showed signs of infection. Mice receiving the 18 per cent casein diet, as usual, responded with unmistakable paralysis.

A comparison of the death rates of the controls and virus-inoculated deficient mice (figure 1) again suggests that the mice may have died from virus infection, since very few of the controls had died when the inoculated groups had reached 90 to 100 per cent mortality. If this series is compared with series 93, it is evident that all virus-inoculated groups in series 97 died faster than in series 93,

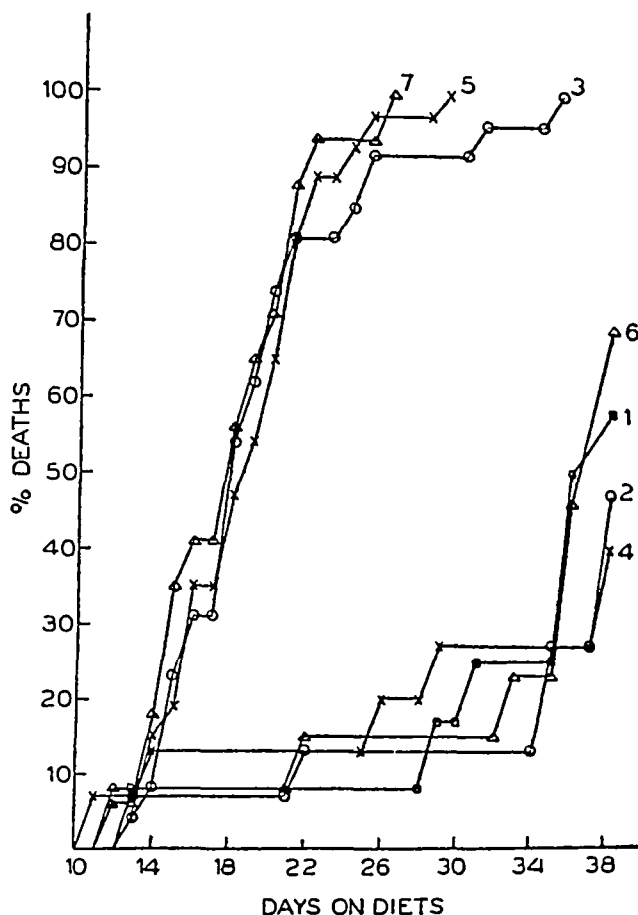


FIG 1 DEATH RATES OF TRYPTOPHANE-DEFICIENT MICE (Series 97)

Groups 1, 2, and 3 were tryptophane deficient groups (diet no 11) group 1 was not inoculated, group 2 was inoculated with normal brain material, group 3 with virus. Groups 4 and 5 were tryptophane-deficient plus double vitamins (diet no 13) group 4 was inoculated with normal brain material and group 5 with virus. Groups 6 and 7 had an acid hydrolyzed casein diet (diet no 14) group 6 was inoculated with normal brain material, group 7 with virus.

including those on the rations containing tryptophane, although control mice died less rapidly than in series 93. This supports the view that the mice died from the effects of the virus, the faster death rate probably being due to a more potent virus inoculum in this series. Again a delay is apparent in deaths in deficient groups compared to those receiving tryptophane, though not so marked as in series 93. Sizable differences in incidence exist, however, through the

fourteenth day after inoculation in group 3, the thirteenth in group 5, and the twelfth day in group 7, compared to the optimum mice

The deficiency signs in this experiment were similar to those seen in previous series. In the virus-inoculated groups deficiency signs appeared earlier and became severe sooner than in the control deficient, as evidenced especially by the time of appearance of tonic convulsions. It was increasingly apparent in this experiment that when tonic convulsions made their appearance, the deficiency was acute and the mouse would die within several days. Many of the

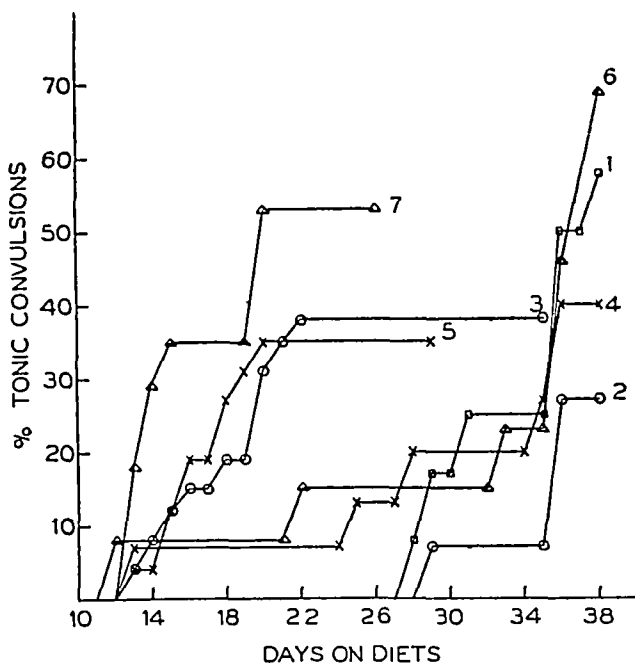


FIG 2 PERCENTAGE OF TONIC CONVULSIONS IN TRYPTOPHANE-DEFICIENT MICE PLOTTED AGAINST TIME (Series 97)

Groups 1, 2, and 3 were tryptophane deficient groups (diet no 11) group 1 was not inoculated, group 2 was inoculated with normal brain material, group 3 with virus. Groups 4 and 5 were tryptophane-deficient plus double vitamins (diet no 13) group 4 was inoculated with normal brain material, group 5 with virus. Groups 6 and 7 had an acid-hydrolyzed casein diet (diet no 14) group 6 was inoculated with normal brain material, group 7 with virus.

deaths seemed to be direct results of the convulsions, since mice which had undergone several of these were often found dead in this position. Figure 2 indicates the percentage of mice in the various deficient groups experiencing tonic convulsions, the time referring to the day of the first such convulsion in a given mouse. These convulsions made their appearance as early as the thirteenth day on the diet, or 5 days after inoculation, and reached a maximum around the twenty-first day in group 3. By this time most of these mice were dead and the percentage did not increase further. Tonic convulsions began in the control groups after about 24 to 28 days on the diets and steadily increased in frequency.

until the experiment was terminated on the thirty-eighth day. It should be noted that no table or figure can tell the whole story. Although we have succeeded in eliciting convulsions in many of the mice and have examined them as frequently and carefully as possible, a few of the mice have been observed in convulsions without the stimulation of spinning, and several have been found dead in the position of the convulsion without previous history of such signs. For this reason the final percentages indicated on figure 2 cannot be taken as the total number of mice undergoing tonic convulsions, and the curves merely indicate a trend in the time period in which convulsions are seen. This trend, however, is definite, and there is no question in our minds that severe signs of deficiency occurred earlier in the mice that received virus inoculations. The general picture presented in these experiments can best be described as a precipitation of the tryptophane deficiency by virus infection, with resultant death of the mice without signs of infection. The direct cause of death then may be either the virus or the deficiency or both, and the indirect cause of death from this point of view would be the virus.

In order to investigate this concept, titrations for virus content were performed using brains and cords from mice in all of the virus-inoculated groups. For these titrations brains and cords were removed from the mice as soon after death as possible and stored in 50 per cent glycerol-saline solution until all the necessary brains were collected and could be titrated simultaneously. To determine the amount of virus which would have been present from the inoculation alone, before multiplication could take place, a "blank" titration was performed, using three brains and cords from the deficient mice, 1 from each group, which were sacrificed about 2 hours after inoculation. These were also preserved in glycerol-saline solution until they could be titrated with the others. Material from the deficient mice was grouped according to the time of death and the signs and symptoms shown, several brains and cords being pooled for each titration. Each pool of brains and cords was ground with alundum and diluted with broth to make 10 per cent suspensions of the original weight of tissue. These were then centrifuged lightly to remove the tissue particles, and 10-fold dilutions of the supernatants were made in broth as indicated for each titration. Three mice were injected intracerebrally under light ether anesthesia with 0.03 ml for each dilution. The mice used were from our own colony and were placed on this experiment as for one of the nutritional series, using the split litter technique. They were fed the 18 per cent casein optimum diet (diet no. 1) and inoculated with the various suspensions when 26 to 31 days old, according to the date of birth. The materials and dilutions used in the titrations are listed below.

(1) "Blank titration." Three brains and cords from deficient mice as described above were pooled and injected at 5 per cent, 1 per cent, and 0.1 per cent dilutions.

(2) Brains and cords were pooled from five optimum mice in group 10 that had become paralyzed in 5 to 7 days and were allowed to die (6 to 8 days) before the brains were removed. The dilutions injected were  $10^{-2}$  through  $10^{-4}$ .

(3) Brains and cords were pooled from four deficient mice in group 3 that had



died 6 to 9 days after inoculation, with only deficiency signs and no signs of infection. The dilutions injected in this and the rest of the titrations were  $10^{-2}$  through  $10^{-5}$ .

(4) Brains and cords were pooled from four deficient mice in group 5 that had died without signs of infection in 6 to 9 days.

(5) Brains and cords were pooled from three deficient mice in group 7 (acid-hydrolyzed casein diet) that had died without signs of infection in 6 to 9 days.

(6) Brains and cords were pooled from two deficient mice in group 3 that had died without signs of infection in 10 to 13 days after inoculation.

(7) Brains and cords were pooled from three deficient mice in group 7 that had died without signs of infection in 10 to 13 days.

(8) Brains and cords were pooled from three deficient mice, one from group 3 and two from group 5, that had died without signs of infection in 10 to 13 days.

(9) Brains and cords were pooled from three deficient mice, two from group 3 and one from group 5, that were listed as positive virus infection and died in 14 to 17 days.

(10) Brains and cords were pooled from three deficient mice in group 3, two had died without signs of infection, one was listed as positive virus infection, and all three had tonic convulsions with death occurring in 10 to 13 days.

(11) Titration from group 8, acid-hydrolyzed casein diet, with tryptophane added. Four brains were pooled from mice that had become paralyzed and died in 6 to 8 days.

(12) Titration from group 9, 8.4 per cent EAA diet (containing tryptophane). Three brains were pooled from mice that had died in 6 to 8 days, one had been paralyzed, one obviously had a virus infection without paralysis, and one had died without signs of infection.

The results of these titrations are presented in table 6. Unfortunately, not enough mice were available to carry out these titrations further, and the end points cannot be stated with certainty. So far as the optimum titration is concerned, it is reasonably certain that the end point was nearly reached. This virus has seldom produced infection at dilutions of  $10^{-7}$  and  $10^{-8}$  in even one of three mice. For the titrations of deficient brains we have, of course, no previous basis. If the incubation periods are taken as an indication, then the end points were nearly reached, since in all titrations the time of paralysis lengthened from 5 to 6 days at the  $10^{-2}$  dilution to 11 and 12 days with the  $10^{-5}$  dilution. With these points and the fact that blank titration produced negative results in mind, it is apparent that the virus multiplied in the deficient mice to approximately the same degree as in optimum mice, regardless of the signs shown by the mice. The course of the disease in all mice in these titrations was typical.

*Controls for the presence of other viruses.* In general, irritability, tonic convulsions, and similar signs are referable to lesions of the brain, the causes of which may differ widely. Since other viruses that produce central nervous system lesions can also produce the signs and symptoms seen in our deficient mice, the possibility of a latent virus activated by the deficiency had to be considered, although we have had no suggestion of such in our mouse colony. Pinkerton

and Swank (1940) reported one instance in which thiamine deficiency allowed a latent psittacosis virus to multiply and produce signs of infection in pigeons. Particularly, the virus of lymphocytic choriomeningitis (LCM) and Theiler's FA virus can produce tonic convulsions of the sort we have described for mice on the tryptophane-deficient diets. In fact, the original paper of Theiler and Gard (1940) on the FA virus describes tonic convulsions caused by this virus in complete resemblance to those we have seen, with the sole exception that in tryptophane deficiency such convulsions are not accompanied by the other encephalitic signs prominent in FA infection. We attempted to test for a latent virus in two ways. First, two guinea pigs weighing 350 to 400 g were inoculated with the brain and cord suspension used in titration no. 10, described above. One pig received 0.1 ml intracerebrally and the other 1.0 ml subcutaneously. These animals have remained healthy, indicating that LCM virus was probably

TABLE 6  
*Titration of brains and cords of mice from series 97*

TITRATION NO	DILUTIONS INJECTED, RESULTS*					
	5%	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
1	0/3	0/3	0/3	—	—	—
2	—	3/3	3/3	3/3	3/3	2/3
3	—	3/3	3/3	3/3	3/3	—
4	—	3/3	3/3	3/3	2/3	—
5	—	3/3	3/3	3/3	2/3	—
6	—	3/3	3/3	3/3	3/3	—
7	—	3/3	3/3	3/3	2/3	—
8	—	3/3	3/3	3/3	0/3	—
9	—	3/3	3/3	2/3	0/3	—
10	—	3/3	3/3	3/3	3/3	—
11	—	3/3	3/3	3/3	3/3	—
12	—	3/3	3/3	3/3	3/3	—

\* Numerator = no. of mice developing infection

Denominator = no. of mice injected

absent from this preparation. A third pig, injected with known LCM virus, succumbed to this infection.

The second procedure was the passage of brains from uninoculated tryptophane-deficient mice into young (24-day-old) mice from another stock.<sup>4</sup> The deficient mice used as the source of brains were part of a more recent experiment to be published at a later date. They were, however, our own stock of mice and were fed the same diet (diet no. 11), and the results with these mice did not differ from those reported in the present communication, in both the control and virus-inoculated groups. Three groups of brains and cords were injected into normal mice. One group consisted of three tryptophane-deficient mice, un-

<sup>4</sup> Webster Swiss mice supplied by the Department of Veterinary Science, University of Wisconsin, through the courtesy of Dr. G. K. L. Underbjerg.

inoculated, that had developed signs of severe deficiency and had undergone two or more tonic convulsions. One died on the twentieth day on the ration and the other two were sacrificed. The brains and cords of the three were removed, ground with a muller in the fresh, unglycerinated state, and a 5 per cent suspension was made in broth. Five mice were then injected intracerebrally with 0.03 ml of the 5 per cent suspension. The second group consisted of two mice in an advanced stage of deficiency that had shown tonic convulsions on the twenty-sixth and twenty-ninth days on the diet. These were sacrificed on the thirtieth day and the brains and cords prepared as described for the first group. The 5 per cent suspension was injected intracerebrally into five mice. The third group consisted of two mice that were markedly deficient but in which tonic convulsions had not been observed. One died after 29 days on the diet and was kept until the next day in the refrigerator. The second was sacrificed on the thirtieth day, and the two brains and cords were pooled and prepared as in the other groups. Five mice were then injected with the 5 per cent suspension. In addition, bacteriological cultures were made of the heart blood of these seven mice and of the three brain suspensions in thioglycolate broth and on agar plates containing 5 per cent defibrinated sheep's blood. No microorganisms were found after 1 week's incubation, and the cultures were therefore considered bacteriologically negative. Although no bacteriological studies were conducted on mice in the series reported here, the dead mice in series 97 were autopsied routinely, and no gross pathology was noted.

The mice injected with brains and cords from these uninoculated deficient mice remained healthy for 28 days, at which time they were sacrificed, and it appears unlikely that a latent virus was responsible for the signs we have described for tryptophane deficiency.

*Histological studies* Histological studies (formalin fixation, gallocyannin stained) of a small number of the mice showed no direct correlation between the severity of the lesions and the observed signs. Encephalitic, but not poliomyelitic, lesions were seen, however, in some of the animals that died without showing typical signs of infection.

#### DISCUSSION

Although there are now many reports in the literature of the various effects aberrant nutrition can have on virus diseases, we have not found any report in which the virus infection affects nutrition in any specific fashion. The results we have described here seem to depend both on the effect nutrition may have on the virus disease and on the effect the virus may have on nutrition. Tryptophane deficiency has resulted, in our experience, in a modification of the course of the disease, to the extent that the usual signs of infection are completely depressed in the majority of animals. The infection can scarcely be considered more fulminating in the deficient mice, in which death supervened without signs, since the time of death was later than in mice receiving tryptophane. Although the disease was inapparent up to the time of death, the virus had none the less multiplied, as demonstrated by titrations of the brain and spinal cords. No

attempt was made to compare the rates of multiplication of the virus in mice lacking or receiving tryptophane, but at the time of death the virus had multiplied to about the same degree in deficient mice as in mice on diets adequate in tryptophane, regardless of whether signs of infection or only signs of deficiency had been observed. Histological studies also suggested virus multiplication, since encephalitic lesions were present in brain sections of some of the mice that had not shown signs of infection. Histological examinations extensive enough to determine how frequently such lesions might be present were not possible under our experimental conditions.

The virus, on the other hand, seemed to precipitate the deficiency of tryptophane insofar as the described deficiency signs can be taken as evidence. Signs of advanced deficiency commonly appeared earlier in mice that had received virus, but these same signs appeared later in mice that were not inoculated or that had received injections of normal brain suspensions. Extra vitamin supplements and the presence of the nonessential amino acids apparently had no effect. When tryptophane was present, however, even in a concentration as low as 0.3 per cent of the diet, the results were reversed, deficiency signs were not evident and the majority of mice exhibited the characteristic signs of infection in its usual course, with death following encephalitis and paralysis. The fact of virus multiplication, the presence of encephalitic lesions in some of the mice, the lack of paralysis or clear encephalitic signs, and the early appearance of severe deficiency signs suggest the possibility that death in these mice may have resulted from a combination of deficiency and viral effects, before more characteristic encephalitic signs and paralysis could develop.

Since the virus had multiplied in the deficient mice, and it seems not unlikely that tryptophane is a constituent of the virus, it is possible that virus multiplication rendered residual tryptophane unavailable to the host, thus making the deficiency signs more acute.

Preliminary experiments indicate that a similar situation obtains in methionine and valine deficiencies with this same virus. Tonic convulsions have also been observed in methionine deficiency, but not as yet in a deficiency of valine. It may be that some necessary metabolic component cannot be synthesized when certain of the essential amino acids are not available in sufficient quantity.

The importance of inanition in the results we have obtained is difficult to assess. It is true that mice on these rations eat very little. We have studied the effects of inanition on this virus disease and have ascertained that reduction of the total food intake to 1 g per day, or caloric restriction to 1 g of the fats, proteins, salts, and vitamin mixture, plus only 0.5 g of sucrose does not duplicate the results with tryptophane deficiency. Typical signs of infection were somewhat suppressed in about half of the mice, but there was no delay or reduction in incidence. Many of the deaths occurred before paralysis began in control groups fed the complete diet ad libitum and might be considered more fulminating infections. Attempts to reduce the food intake further have resulted in the death of too many of the mice to conduct an effective study. The inanition studies were conducted with the 18 per cent casein diet. This has not been

repeated with the essential amino acid diet since those mice that were fed this ration, including 0.3 per cent tryptophane, also ate very little. The weights are barely maintained and there is often an initial weight loss, not so marked as on the deficient diet. Despite the low food intake of mice on this ration, the signs of infection were clear, uninoculated controls showed no signs of deficiency and survived throughout the experiment.

The observations reported here are seemingly at variance with those of Jones *et al.* (1946) in their study of low tryptophane diets and poliomyelitis. They found that diets low in tryptophane produced a marked delay in onset but no other modification of the disease. They were using, however, a different virus, the Lansing strain of poliomyelitis, and tryptophane was supplied at 0.02 per cent level in an otherwise deficient diet containing zein as the protein source. This amount was supplied in order to prevent an undue number of animals from dying of deficiency, a necessary precaution when dealing with a virus that may have a long incubation period. Weight curves given for their mice indicated that this amount of the amino acid was enough to maintain the animals at fairly constant weight. Inasmuch as our deficient diet contained no tryptophane and steady weight losses occurred, the two series of experiments are not strictly comparable.

Since in our experiments the deficient diet seemed to suppress paralysis more effectively than encephalitis, both of which occur in this virus disease, it has appeared worth while to study this deficiency with WEE virus, which produces mainly encephalitis with little cord involvement, and with Theiler's original (TO) virus, which produces poliomyelitis with almost no encephalitic signs. Experiments along these lines are under way and will be reported at a later date. It also appears important to approach this problem from another point of view—the use of amino acid analogues, which may be metabolic antagonists. The study of bacterial viruses has been notably successful with this approach. Tryptophane is apparently necessary for the multiplication of the T2 bacteriophage in *Escherichia coli*, since the analogue 5-methyl tryptophane inhibits the synthesis of virus without affecting the respiratory activity of the host cell, and this inhibition can be reversed specifically by the addition of tryptophane (Cohen and Fowler, 1947). Amino acid analogues have also inhibited the growth of vaccinia virus in Maitland type tissue cultures (Thompson, 1947). The amino acids for which analogues were used in this case were glycine, valine, phenylalanine, and methionine.

#### SUMMARY

Low protein (9 per cent casein) and high protein (36 per cent casein) diets have, in our hands, exerted no influence on infection of mice with Theiler's GDVII virus.

A marked effect on this disease has, however, been produced with diets deficient in tryptophane. The deficiency has been induced by feeding pure amino acid diets (containing only the essential amino acids) minus tryptophane and by feeding acid-hydrolyzed casein, in one series double amounts of vitamins were also

given in an attempt to rule out possible complications in the deficiency. Regardless of the diet used to produce the deficiency, or of the presence of extra vitamins and the nonessential amino acids, the results have been an accelerated death rate compared to control deficient, a delayed death rate compared to those mice receiving the same diets plus 0.3 per cent tryptophane or the 18 per cent casein optimum diet, and a lack of the characteristic signs of infection in the majority of deficient mice. The signs shown by these animals are, however, characteristic of the deficiency but appear earlier than in uninoculated controls, the effect seeming to be a precipitation of the deficiency by virus infection. That the virus had multiplied in the deficient mice in which no signs of infection could be observed was demonstrated by titration of their brains and cords in young normal mice. Histological studies confirmed this, since evidence of encephalitis was found in such animals. Mice receiving tryptophane, added to the purified amino acid or acid-hydrolyzed casein diets, or those on the 18 per cent casein diet, showed typical encephalitis and progressive paralysis. On the amino acid diet paralyzes were frequently fewer than on the 18 per cent casein diet, but infection was indicated by encephalitis when paralysis was absent.

Tryptophane deficiency alone has produced definite signs. Among these is a convulsion of the tonic type, during which the hind legs of the mouse are completely extended, the forelegs flexed, and the whole mouse is extremely rigid and cyanotic. At times the hind legs are extended and the forelegs move convulsively. These convulsions occur in late stages of the deficiency, since death usually follows within several days. Death may occur in one of these seizures, but often the mice undergo such convulsions 3 to 5 times on as many days before dying. The possibility that a latent virus is responsible for these signs has been tested by passage of the brains and spinal cords of these mice intracerebrally into young normal mice and intracerebrally or subcutaneously into guinea pigs. No evidence of an infectious agent was found.

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# THE APPARENT INVOLVEMENT OF *VIBRIO FETUS* IN AN INFECTION OF MAN<sup>1</sup>

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Many articles can be found in the literature concerning the infection of livestock by *Vibrio fetus*. References have been made to such infections in both the male and female animal. To the knowledge of this writer, however, only one report has been made concerning infections with this organism, or at least a very similar one, in the human race. Curtis (1913) reported the isolation of a curved, motile, anaerobic bacillus from uterine discharges of two female patients—one whose infection followed instrumental abortion, and the other whose labor at full term was complicated by the same organism. This rod Curtis assigned to the genus *Vibrio*. Apparently no reports have been made of a vibronic infection in the male human involving this or any very similar organism.

Recently a male worker in this laboratory, in an attempt to obtain a pure culture of a *Staphylococcus* for addition to the stock culture collection, had occasion to open aseptically a small, simple pustule located on the cheek. From the pus and exudate obtained, nutrient agar and blood-enriched (10 per cent) nutrient agar plates were inoculated by the streak method. At the same time a film was made from this material, which was stained by Gram's method. This preparation showed "comma" or "S-shaped" gram-negative organisms, a few short gram-negative rods, and large, nonsporulating, gram-positive rods.

Culturally the large rod grew quite as well on plain nutrient agar as on the blood medium under aerobic conditions and at a temperature of 37 C. Growth was visible in 24 hours. The small, curved, gram-negative rods, however, were reluctant to grow even upon the 10 per cent blood agar. Transfer to a fresh plate of blood agar under an atmosphere of 10 per cent CO<sub>2</sub>, however, produced an increased amount of growth in 3 to 4 days. Colonies of the large rod were 5 to 15 mm in diameter, raised, concave, glistening, smooth, entire, and dirty-white in color. Colonies of the small, curved rod were 1 to 3 mm in diameter, raised, concave, glistening, smooth, entire, and blue to blue-gray in color. Growth of this second organism was also obtained upon inoculation of thioglycolate blood broth.<sup>2</sup> This growth was noted to occur at a depth of one-half inch. To one set of thioglycolate blood broth tubes, 3 per cent gelatin was

<sup>1</sup> The investigation reported in this paper is in connection with a project of the Wyoming Agricultural Experiment Station and is published by permission of the Director.

<sup>2</sup> This consisted of

Neopeptone	10 g	Gelatin	1 g
K <sub>2</sub> HPO <sub>4</sub>	2 g	Meat extract	3 g
Sodium thioglycolate	1 g	Water	1,000 ml
Agar	1 g	Adjusted to pH 7.0	

added. The curved rods did not bring about liquefaction of the gelatin within 1 week. To three other sets of thioglycolate blood broth tubes, glucose, sucrose, or lactose in 1 per cent concentrations was added. According to tests with Benedict's quantitative reagents, these sugars had not been fermented in 1 week although ample growth of the curved rods was noted. Hanging drop examinations of each organism showed them to be motile.

As the cultures aged, the large gram-positive rod was noted to assume the gram-negative reaction to a certain extent. The short, curved rod exhibited a tendency to form short, straight, or "S-shaped" chains. At all ages this organism showed definite granulation with the ordinary stains.

The serological studies upon this isolated curved rod were brief but of interest. A saline suspension of 1 billion cells per cubic centimeter reacted with a serum diluted to 1:120. This serum had previously been drawn from a pregnant ewe infected with a known strain of *Vibrio fetus* (Cambridge ovine strain IV) and had been found to agglutinate that organism in dilutions of 1:160.

This slight infection in itself would have been no cause for interest had it not been for the fact that the infected worker had been engaged in research involving *Vibrio fetus* for some weeks. There is a very definite possibility that the larger gram-positive rod encountered was responsible for the small lesion with the smaller organism restricted to the role of a secondary invader of little or no importance. It is worthy of note, however, that *Vibrio fetus* is capable of maintaining itself within the male human body.

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# STUDIES ON CERTAIN BIOLOGICAL CHARACTERISTICS OF MALLEOMYCES MALLEI AND MALLEOMYCES PSEUDOMALLEI

## I MORPHOLOGY, CULTIVATION, VIABILITY, AND ISOLATION FROM CONTAMINATED SPECIMENS

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The etiological agent of glanders, or farcy, was first isolated in pure culture by Loeffler and Schutz in 1892. The genus *Malleomyces* includes only two species—*Malleomyces malleri* and *Malleomyces pseudomalleri* (Bergey *et al.*, 1939). Very few studies have been conducted with *M. malleri* in the last 25 years since, by the destruction of infected animals detected through mallein skin tests and serum complement fixation tests, the disease has been almost eradicated in most civilized countries from its natural equine hosts.

*M. pseudomalleri* (*B. whitmori*) was identified by Whitmore (1912) as the etiological agent of melioidosis, a disease of man similar to glanders. The wild rat of southeastern Asia is reported to be the natural host, but natural infection has been found in rats, rabbits, cats, and dogs (Stanton and Fletcher, 1932).

The purpose of this paper is to present studies on morphology, cultivation, viability, disinfection, and isolation of the organisms from contaminated specimens. Studies on virulence and animal susceptibility are reported in the succeeding paper. Reports of studies on comparative serological reactions, chemotherapy, chemical fractions, pathological changes in infected animals, and six human cases of glanders will be reported elsewhere.

Eight strains of *M. malleri* and two strains of *M. pseudomalleri* were used in the studies reported. The source, date of isolation, and reported virulence of each strain are given in table 1.

### MORPHOLOGY

The strains of *M. malleri* and *M. pseudomalleri* used in this work were indistinguishable morphologically. Pleomorphism was not marked even in old cultures. Apparent filamentous forms on careful study appeared to be long chains of bacilli that were closely united. When stained with common granule stains, the organisms showed scattered areas of increased density, giving a definite granular appearance. This was especially marked in direct films from infected tissues stained with Wright or Giemsa stains. Study of such preparations gave

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the impression that the organisms were encapsulated, but this could not be proved by the use of any of the common capsule stains. Flagellar stains showed that *M. pseudomallei* possessed lophotrichate flagella, whereas *M. mallei* was atrichous. Broth cultures of *M. pseudomallei* continued to show active motility after storage for 5 months.

When photographed in the electron microscope,<sup>2</sup> the organisms of both species showed marked variations in the internal structure of the cell. In some organisms there appeared to be only a bipolar increase in protoplasmic density. In

TABLE 1  
Data on the strains of *M. mallei* and *M. pseudomallei* studied

ORGANISM	DESIGNATION	SOURCE	DATE OF ISOLATION	REPORTED VIRULENCE	DESIGNATION IN THIS PAPER
<i>M. mallei</i>	2024-2MP*	Unknown	Unknown—very old	Low	2MP
<i>M. mallei</i>	2024-3PP*	Unknown	Unknown—very old	Low	3PP
<i>M. mallei</i>	2024-3MP*	Unknown	Unknown—very old	Low	3MP
<i>M. mallei</i>	3†	Kweiyang, China	1942	Straus-positive	C3
<i>M. mallei</i>	4†	Lung of horse	1942	Straus-positive	C4
<i>M. mallei</i>	5†	Lung of horse	1942	Straus-positive	C5
<i>M. mallei</i>	K†	Unknown	Unknown	Unknown	C6
<i>M. mallei</i>	3873*	Fatal human case in China	1944	Very high	C7
<i>M. pseudomallei</i>	294†	Unknown	Unknown	Avirulent	W294
<i>M. pseudomallei</i>	295†	Unknown	Unknown	Avirulent	W295

\* Cultures from U. S. Army Veterinary Laboratories used in the preparation of mallein. The authors are indebted to Colonel R. Randall, VC, USA, for supplying these strains.

† Type cultures from the China Epidemic Prevention Bureau (Dr. T'anga's laboratory). The authors are indebted to General R. A. Kelser, VC, USA, for procuring these strains.

‡ Old laboratory stock cultures from the Calcutta School of Tropical Medicine. The authors are indebted to General R. A. Kelser, USA, for procuring these strains.

others, the cell contained scattered, clear, refractile bodies resembling lipoidal globules and opaque bodies resembling dense protoplasmic accumulations (figures 1, 2, and 3). A definite cell wall was visible, and flagella were scattered through the field surrounding the cells of *M. pseudomallei* (figure 3). Electron micrographs of *M. pseudomallei* taken after a suspension was subjected to ultrasonic vibration for 1 hour showed numerous small circumscribed bodies in the cellular debris, resembling extruded lipoid globules (figure 4). Fat bodies within the cells of *M. mallei* have been demonstrated previously by special fat stains (We

<sup>2</sup> We are indebted to Dr. L. A. Chambers for the preparation of the electron micrographs.

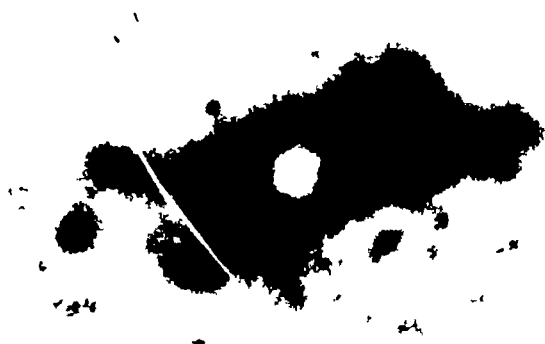


Fig 1 ELECTRON MICROGRAPH OF *M. MALLEI*

Note areas of increased protoplasmic density, light areas resembling vacuoles, and refractile cell membrane. Magnification approximately 27,000  $\times$



FIG 2 ELECTRON MICROGRAPH OF *M. MALLEI* SHOWING DENSE GRANULES AND CLEAR REFRACTILE AREAS WITHIN THE CELLS  
Magnification approximately 13,500  $\times$



FIG 3 ELECTRON MICROGRAPH OF *M. pseudomallei*

Note intracellular refractile areas, areas of increased density, and flagellar strands scattered around the cells. Magnification approximately 12,500  $\times$



ley and Young, 1945) and a complex lipoidal substance was extracted chemically from the organism in this laboratory (Hink, Miller, and Tanner, 1948)

#### CULTIVATION

The medium used routinely for *M. mallei* by former workers was a meat infusion base to which 1 per cent glycerol and 1 per cent peptone were added. *M. pseudomallei* was known to grow well on standard laboratory media. In an effort to find simple base media and synthetic media that were satisfactory for both organisms, the growth of both organisms on the test media was carefully compared with that on the glycerinated meat infusion peptone media. Comparison was made by plate counts (surface-streaking technique) and colonial study on solid media, and by turbidity measurement and viability counts of liquid media cultures. The optimum pH for both organisms was 6.8. The following media (all contained 4 per cent glycerol and were adjusted to pH 6.8) supported growth of both organisms, equal in every respect to the infusion base medium.

- (a) Beef extract (0.3 per cent) peptone (1 per cent) agar
- (b) Potato infusion agar (no beef or peptone)
- (c) Cysteine (0.1 per cent) peptone (1 per cent) agar
- (d) Asparagine (0.5 per cent) agar (no beef or peptone)
- (e) Beef extract (0.3 per cent) peptone (1 per cent) broth

*M. pseudomallei* also grew well on all standard bacteriological media and did not require glycerol, although growth was somewhat enhanced by its presence. *M. mallei* required glycerol for optimum growth.

The colonial appearance and biochemical activity of the strains of *M. mallei* studied corresponded with those described by previous workers. The colonial appearance of both strains of *M. pseudomallei* was the same as that described by Stanton and Fletcher (1932), but our strains did not actively ferment any of the sugars tested. Colonies of *M. mallei* on agar were smooth, entire, convex, translucent, and similar to those of *Escherichia coli* except that the latter were somewhat larger. *M. pseudomallei* colonies were smooth after 24 hours' incubation and were identical in appearance with 48-hour *M. mallei* colonies. They became rough and flat and had finely crenated margins after 48 to 72 hours' incubation. Mucoid and intermediate colonial types of *M. pseudomallei* (Stanton and Fletcher, 1932) and rough variants of *M. mallei* were observed from time to time, but serial colonial isolation gave only 75 to 90 per cent of the colonies in the variant phase, and on continued cultivation all reverted to the original type.

The distinctive yellowish-brown pigmentation of *Malleomyces* organisms did not appear until after 4 to 7 days' incubation. *M. pseudomallei* showed rapid and complete proteolytic activity on all media containing colloidal or coagulated proteins.

Since it was observed that both organisms grew well on agar containing 0.5 per cent asparagine as the only source of nitrogen, synthetic media which contained asparagine as the source of nitrogen were chosen for study. Luhrs' (1927)

modification of Long's synthetic medium for *Mycobacterium tuberculosis* was found to support good growth of both organisms and the total bacterial counts were equal to those in the infusion base broth

**Aeration and oxygenation** Since *Malleomyces* organisms are obligate aerobes and produce heavy growth on the surface of media, studies were undertaken to determine the effect of increasing the oxygen tension in broth cultures. Aeration was effected by bubbling air under pressure through broth in sintered glass filter flasks or through Mandler filter candles in Erlenmeyer flasks of broth. Oxygenation was accomplished in like manner using pure oxygen under pressure. A thin layer of mineral oil or pure lard was placed on the surface of the medium to prevent foaming. The effluent from the sealed flasks was passed through a series of large cotton filters to prevent the liberation of infectious aerosols into the laboratory air. Cultures were incubated at 37 C until maximum growth had occurred (3 to 5 days), and aliquot portions were removed for plate counts.

Aerated cultures of *M. mallei* were homogeneous heavy suspensions without pellicle formation. Although cultures of *M. pseudomallei* showed moderate pellicle formation, the broth was cloudy throughout, and the amount of pellicle was much less than in nonaerated cultures. The maximum viability counts on aerated cultures of both organisms varied from  $1 \times 10^9$  to  $5 \times 10^9$  organisms per ml, compared with  $8 \times 10^7$  to  $2 \times 10^8$  organisms per ml in nonaerated cultures. Oxygenation produced maximum counts of  $8 \times 10^9$  to  $2 \times 10^{10}$  organisms per ml.

#### DISINFECTION

Several of the strains (C3, C4, C5, C7, and W294) of *Malleomyces* studied were highly infectious for animals, and *M. mallei* proved to be infectious for man (Howe and Miller, 1947). It was of considerable importance, therefore, to determine the relative efficacy of common disinfectants against these organisms. Tests were made on sodium hypochlorite, phenol, "roccal" (benzalkonium chloride), tincture of iodine, lysol, mercuric chloride in alcohol, and potassium permanganate. One-tenth ml of a saline suspension containing 50 million organisms was added to 5 ml of each disinfectant solution. At intervals of 5, 10, 15, and 30 minutes, 0.2 ml of the organism-disinfectant mixture were withdrawn and inoculated into 10-ml broth tubes. In testing hypochlorite solutions, 0.01 per cent ammonium thiosulfate was added to the broth tubes to neutralize the hypochlorite carried over from the test mixture. The broth was observed for turbidity after 72 hours' incubation, and 0.1 ml of the broth was plated out as a final test for viability.

Table 2 gives the concentrations of the disinfectants tested and the minimum time necessary for sterilization of the suspension of organisms. Hypochlorite (500 ppm available chlorine), "roccal" (1:2,000), iodine, mercuric chloride in alcohol, and potassium permanganate were highly effective. Phenol was less effective and lysol was ineffective. "Roccal" (1:2,000) was chosen for routine use because of its high disinfectant action and its lack of corrosive and irritating action.



## PRESERVATION OF VIABILITY AND VIRULENCE

The preservation of the viability and the virulence of the various strains was investigated, since the literature gave little information on this subject and no reference to the use of lyophilization was found

*Viability and virulence in stored cultures* Agar and broth cultures of *M. mallei* and *M. pseudomallei* remained viable for 2 to 3 months when stored at room temperature (22 to 28 C), but died more rapidly when stored in the 37 C incubator, in the refrigerator (3 to 5 C), or in the frozen state (minus 10 to 30 C). Although still viable, cultures of *M. mallei* showed a marked drop in viru-

TABLE 2

*The germicidal activity of common disinfectants on M. mallei and M. pseudomallei*

DISINFECTANT SOLUTION	TIME IN MINUTES NECESSARY TO KILL	
	<i>M. mallei</i>	<i>M. pseudomallei</i>
Sodium hypochlorite		
500 ppm chlorine	5	5
100 ppm chlorine	30	15
Phenol 1%	15	15
5%	15	10
"Roccal" 1 2,000	5	5
1 5,000	>30	30
Tincture of iodine (7%)		
1 10	5	5
Lysol 1%	>30	>30
3%	>30	>30
Mercuric chloride (1% in 35% ethyl alc.)	5	5
Potassium permanganate (1% in 1% HCl)	5	5

lence for experimental animals after storage for 4 to 6 weeks at any of the temperatures mentioned. When the cultures of *M. mallei* were transferred weekly or biweekly, virulence remained constant for 2 to 3 months. Virulence was kept at a constant level by transferring stock cultures weekly and passing each strain through hamsters once a month. *M. pseudomallei* (virulent strain W294), however, retained a constant degree of virulence for experimental animals without animal passage, even when stored in culture for 2 to 3 months.

Lyophilization preserved the viability and virulence of both organisms for relatively long periods. Heavy suspensions of organisms were prepared by emulsifying the growth scraped from agar plates into sterile inactivated rabbit serum. Horse serum could not be used, since it contained a thermostable component that was lethal for *Malleomyces* organisms. Suspensions were lyophilized

with a portable lyophile apparatus in a refrigerated room held at 0 C. This was found to be essential since all organisms were killed when lyophilization was done with the apparatus in a room at 15 to 20 C. It was apparent that it was necessary to keep the temperature of the frozen suspensions considerably below 0 C to obtain successful lyophilization. The quantitative preservation of viability was determined by plate counts on suspensions before lyophilization and after various intervals of storage. The two species of *Malleomyces* showed about the same degree of preservation. The average of two separate tests with each species showed that with an original count of  $6.9 \times 10^{10}$  organisms per ml, 23 per cent of the cells were viable 24 hours after lyophilization and 8.3 per cent were viable 13 weeks after lyophilization.

In one additional test with *M. pseudomallei* the count after 6 months of storage was  $6 \times 10^7$  compared with the original count of  $6.8 \times 10^9$  organisms per ml. The virulence of *M. mallei* was lowered moderately by prolonged storage in lyophilized culture, but was rapidly restored by one animal passage through hamsters. The virulence of *M. pseudomallei* was not changed after 6 months' storage, as indicated by MLD titrations in hamsters.

TABLE 3  
*Viability of M. mallei and M. pseudomallei in tap water*

TIME	M. MALLEI ORGANISMS PER ML	M. PSEUDOMALLEI ORGANISMS PER ML
At once	$3.5 \times 10^7$	$1.8 \times 10^7$
2 weeks	$4.0 \times 10^7$	$2.0 \times 10^8$
4 weeks	$1.0 \times 10^8$	$4.0 \times 10^7$
5 weeks	0	$7.0 \times 10^8$
8 weeks	0	$1.0 \times 10^9$

*Viability in tap water.* The transmission of glanders in horses has long been known to occur by contamination of water in drinking troughs (Hutyra and Marek, 1926). Several human cases of melioidosis are reported after ingestion or inhalation of lake or pond water (Huard and Long, 1937). The degree of survival of *Malleomyces* organisms in water was studied by making a dilute suspension of the 24-hour growth scraped from the surface of an agar slant culture into sterile tap water. The dilute suspension was stored at room temperature, and triplicate plate counts were made at once and after 2, 4, 5, and 8 weeks of storage. The results given in table 3 show that *M. mallei* survived well for 4 weeks and then decreased in numbers rapidly. *M. pseudomallei* apparently increased in numbers during the first 2 weeks, and a high percentage survived during the 8 weeks of storage. The degree of survival of these organisms in tap water indicates that water sources might remain infectious to animals or man for several weeks after pollution.

#### ISOLATION OF MALLEOMYCES FROM CONTAMINATED SPECIMENS

Because of the marked variation in the clinical signs and symptoms, the diagnosis of acute glanders or of melioidosis in man is largely dependent upon

isolation of the etiological agent. When bacteremia is present, isolation by blood culture offers no difficulties. However, isolation from contaminated biological materials such as exudates, sputum, feces, and urine and from possible natural sources such as food, water, or soil requires special techniques. Studies were undertaken to develop methods of differential cultivation and animal inoculation that would permit the isolation and recognition of these organisms when present in small numbers in contaminated specimens.

*Differential cultivation.* Plate counts were performed on a suspension of one or more strains of each organism on agar plates containing serial dilutions of a number of common inhibitor dyes and on plain glycerinated agar plates. The interpretation of the results was based on the plate counts and the appearance of the growth. A list of suitable inhibitor dyes with the maximum concentration that gave no inhibition of growth of *M. mallei* and *M. pseudomallei* is as follows:

Crystal violet	1 200,000
Proflavine	1 500,000
Acriflavine	1 500,000
Acridine orange	1 500,000
Acridine yellow	1 500,000
Basic fuchsin	1 100,000
Acid fuchsin	1 100,000
Malachite green	1 1,000,000
Brilliant green	1 1,000,000

All of these dyes, in the concentration indicated, inhibited gram-positive organisms. Although any one of the dyes could be used successfully to inhibit gram-positive organisms, crystal violet was chosen for routine use. None of the dyes were suitable for differential inhibition of other gram-negative organisms since concentrations adequate for this purpose also inhibited *Malleomyces* organisms.

Eosin-methylene-blue medium was adapted for use with *Malleomyces* by adding 4 per cent glycerol. *Malleomyces* grew well on this medium and the colonies were clear or slightly bluish. Differentiation from coli-aerogenes colonies was made with ease. The limitations of this method for isolation of *Malleomyces* from fecal suspensions were (1) coli-aerogenes colonies become comparatively large in the 48-hour incubation period necessary for the development of *M. mallei*, and (2) the lack of any significant inhibitory action of this medium allows overgrowth of gram-positive as well as gram-negative contaminants in all except the higher dilutions of the specimen.

*Rapid colonial identification tests.* Studies were undertaken to find methods of rapid identification of suspicious *Malleomyces* colonies on crystal violet agar. The oxidase test (Ellingworth *et al.*, 1929, Gordon and McLeod, 1928) was found to be positive on *Malleomyces* colonies. Since there was an interval of about 5 minutes between the development of a positive test and death of the organisms, the colonies giving a positive test could be subcultured or inoculated into hamsters.

The rapid slide agglutination technique was applied and gave dependable

results for confirming suspected *Malleomyces* colonies. The two species of the genus could not be differentiated by this test, however, because of the serological cross reactions (Cravitz and Miller, 1948).

*Use of antibiotics* Since penicillin had been found to exert no effect on *Malleomyces in vitro* (Miller, Pannell, and Ingalls, 1948), this antibiotic was used to reduce the number of the other organisms in contaminated specimens. Specimens were incubated for 3 hours at 37 C in saline containing 1,000 units of penicillin per ml and were then plated out on crystal violet agar or injected into hamsters. Gram-positive organisms were killed or inhibited and isolation of *Malleomyces* organisms was greatly facilitated.

*Animal inoculation* Saline extracts or suspensions of suspected materials were injected subcutaneously and intraperitoneally into adult male hamsters. For grossly contaminated material, the suspension was incubated with penicillin for 3 hours as described above before animals were inoculated. The development of a Straus reaction, the death of the animal with typical findings at autopsy of acute glanders or melioidosis, and cultivation of the organisms from infected tissues gave proof of the presence of *Malleomyces* organisms in the specimen.

*Application of the methods* In order to test the efficiency of the methods, studies were conducted to determine the lowest proportion of *Malleomyces* to total contaminant organisms that would permit recovery of *Malleomyces*. Normal animal cage sweepings were suspended in isotonic saline solution as a source of contaminants and plate counts were made to determine the number of organisms present. Known numbers of *M. mallei* and of *M. pseudomallei* were added to aliquot portions of this suspension. The cultural isolation technique was tested by inoculating these mixed suspensions on crystal violet agar plates and identifying the colonies of *Malleomyces* by the oxidase reaction and slide agglutination tests. The animal inoculation technique was tested by treating the mixture with penicillin for 3 hours and inoculating aliquot portions subcutaneously and intraperitoneally into hamsters.

It was found that *Malleomyces* could be easily and uniformly recovered by the cultural method when the original proportion of these organisms to contaminants was as low as 1 to 100. Since hamsters were very susceptible to glanders and melioidosis (MLD 15 to 20 organisms), and not very susceptible to infection with the contaminants present, *M. mallei* and *M. pseudomallei* could be uniformly recovered by the animal inoculation method when the original proportion of *Malleomyces* to contaminants was as low as 1 to 12,500.

Application of the methods to feces samples to which *Malleomyces* organisms were added was less successful. EMB agar gave satisfactory differentiation of colonies, but, because of the limitations of the method, the proportion of *Malleomyces* to *Escherichia* and *Aerobacter* organisms had to be larger than 1 to 100 to get uniform recovery. Injections of fecal samples into hamsters frequently produced fatal coli-aerogenes infections unless the specimen was highly diluted.

*M. mallei* and *M. pseudomallei* were isolated from blood, urine, and purulent exudates from infected hamsters and guinea pigs by the foregoing methods. *M. pseudomallei* was isolated from the purulent nasal discharge of infected rabbits. Attempts to isolate *Malleomyces* from feces of infected animals were

successful Application of the methods in six human cases of acute pulmonary glanders failed to reveal the specific organisms in the blood or sputum (Howe and Miller, 1917)

#### DISCUSSION

The intracellular granulations prominent in stained preparations of the organisms and in electron micrographs appear to be lipid globules and accumulations of increased protoplasmic density This is in contrast to the apparently similar granulations in the genus *Corynebacterium*, which are thought to be protein materials within the cells

The vigorous growth and the stability in respect to viability and virulence of *M. pseudomallei* were outstanding characteristics of this organism The fact that both species of *Malleomyces* organisms remained viable for at least 4 weeks, when suspended in tap water, emphasized the importance of ingestion or inhalation of polluted water supplies as a means of transmission of glanders and melioidosis

The two species of the genus *Malleomyces* are very closely related They are indistinguishable morphologically, difficult to distinguish serologically (Cravitz and Miller, 1948), and they produce diseases in experimental animals that are practically indistinguishable clinically and pathologically (Miller, Smith, and Tanner, 1948) The two species differ in only a few respects *M. pseudomallei* is motile, grows in 24 hours on ordinary media, produces predominately rough colonies on agar, has greater proteolytic activity, and tends to produce a more fulminating disease *M. mallei* is nonmotile, requires 48 hours' incubation for growth, and requires glycerol in the culture medium

*Malleomyces* organisms have a number of characteristics that suggest a relationship to *Mycobacterium tuberculosis* The cells contain a complex lipoidal material (Worley and Young, 1945, Hink, Miller, and Tanner, 1948), the organisms grow in a synthetic medium developed for *Mycobacterium tuberculosis*, *M. mallei* requires glycerol in the medium and the growth of *M. pseudomallei* is enhanced by its presence, skin test substances (mallein and whitmorine) are produced that have many of the properties of tuberculin, and the lesions produced are granulomatous in character Furthermore the gross pathological changes in experimental animals show a marked resemblance to those in acute military tuberculosis (Miller, Smith, and Tanner, 1948) The two genera differ in a number of other characteristics, but the similarities are worthy of note

The lack of detailed knowledge of the mechanisms of natural transmission suggests the need for careful studies on the epidemiology of glanders and melioidosis The methods described for isolation of the organisms by differential cultivation and animal inoculation would be of assistance in analyzing the role of animal carriers, insect vectors, and polluted food and water supplies in the transmission of these diseases

#### SUMMARY

Electron micrographs of *Malleomyces* organisms show intracellular refractile bodies resembling lipid globules and opaque areas of increased protoplasmic

density *Malleomyces pseudomallei* possesses lophotrichate flagella, whereas *Malleomyces mallei* is atrichous

Both organisms grew well on beef extract base media. *M. mallei* required the addition of glycerol. Both organisms grew well in Luhrs' modification of Long's synthetic medium for *Mycobacterium tuberculosis*. Aeration and oxygenation of broth cultures produced a heavy growth with an even turbidity and little or no pellicle.

Studies of the efficacy of common disinfectant solutions showed "roccal" (benzalkonium chloride), hypochlorite, iodine, and mercuric chloride to be highly effective. Phenol was less effective and lysol was ineffective.

Viability and virulence of cultures were well preserved over periods of at least 3 to 6 months by lyophilization.

Both organisms remained viable for at least 4 weeks when suspended in ordinary tap water, and *M. pseudomallei* apparently increased in numbers.

Effective methods of isolation of *Malleomyces* organisms from contaminated specimens by differential cultivation, rapid colonial identification, and animal inoculation are described.

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# STUDIES ON CERTAIN BIOLOGICAL CHARACTERISTICS OF *MALLEOMYCES MALLEI* AND *MALLEOMYCES PSEUDOMALLEI*

## II VIRULENCE AND INFECTIVITY FOR ANIMALS

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As a natural infection, glanders occurs only in horses, mules, and donkeys. Other animals occasionally become infected from contact with infected solipeds. The most susceptible of these occasionally infected species are ferrets, moles, field mice, cats, and dogs. Sheep, goats, hogs, rabbits, white mice, and house mice are reported to be less susceptible, and cattle are immune (Hutyra and Marek, 1926). The guinea pig is considered to be the most susceptible laboratory animal. The virulence of various strains of *Malleomyces mallei* has been reported to vary widely (Bernstein and Carling, 1909, Dudgeon *et al*, 1918), but in the virulence tests recorded in the literature large doses were given, and evaluation of virulence was made solely on the severity of disease produced.

The wild rats of southeastern Asia constitute a natural reservoir of melioidosis. The disease, chronic in the rat, is thought to be transmitted to other animals, and to man, by ingestion or inhalation of materials contaminated with rat excreta. Rabbits, dogs, and cats have been found infected naturally, and an occasional case of infection has been reported in other domestic animals. Guinea pigs, mice, and rabbits have been used in studies of the experimentally produced disease (Stanton and Fletcher, 1932). Guinea pigs were reported to be almost universally susceptible, dying of a fulminating infection within 24 hours after a massive inoculation and within 3 weeks after a smaller inoculation. Monkeys were more resistant, developing fatal infection only after ingestion of massive doses. Rabbits and guinea pigs were susceptible to inoculation by the intraperitoneal or subcutaneous routes and by ingestion and inhalation. Although the organisms were reported as highly virulent, the inoculum always contained thousands to millions of organisms (0.01 to 1 ml of a 48-hour broth culture—Stanton and Fletcher, 1932). No reference to a more quantitative evaluation of virulence by MLD or LD<sub>50</sub> determinations was found in the literature.

The present studies include a comparison of the virulence of the several strains and their infectivity by various portals of entry, and a comparison of the susceptibility of several common species of laboratory animals. The source and characteristics of the strains of *Malleomyces* studied are given in the first paper of this series (Miller *et al*, 1948). The pathological changes and pathogenesis

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of experimental glanders and melioidosis will be reported elsewhere (Miller, Smith, and Tanner, 1948)

### COMPARATIVE VIRULENCE OF STRAINS

Since hamsters proved to be the most susceptible of the common laboratory animals tested, the virulence of the several strains was compared in this species. Decimal dilutions of a suspension of the growth from a 24-hour agar slant culture were inoculated intraperitoneally, using 5 to 8 animals for each dilution. The number of organisms injected was determined by triplicate plate count. All animals that died were autopsied and cultures of the infected organs were made. Survivors were held from 20 to 30 days, killed with nembutal, and examined for

TABLE 1

*Comparative virulence for hamsters of strains of M. mallei and M. pseudomallei injected intraperitoneally*

STRAIN	NUMBER OF ORGANISMS IN	
	MLD	LD <sub>50</sub>
<i>M. mallei</i> 2MP	>500,000,000*	
<i>M. mallei</i> 3MP	>500,000,000*	
<i>M. mallei</i> 3PP	>500,000,000*	
<i>M. mallei</i> 2MP (serial passage)	7,000	
<i>M. mallei</i> C3	20	12
<i>M. mallei</i> C4	20	12
<i>M. mallei</i> C5	>900,000*	
<i>M. mallei</i> C6	>1,200	
<i>M. mallei</i> C7†	<26	2‡
<i>M. pseudomallei</i> W294	15	6
<i>M. pseudomallei</i> W295	>85,000,000*	

\* Produced 50 to 90% mortality

† Only one titration was done with this strain, see text

‡ This value is only approximate, calculated dose of 26 organisms produced 80% mortality

the presence of pathologic changes, cultures were made of the liver, spleen, lungs, and heart blood.

The least number of organisms that produced a 100 per cent morbidity and mortality was considered the minimum lethal dose (MLD). When possible, the LD<sub>50</sub> dose was computed. The average results of several titrations are given in table 1.

It will be noted from table 1 that strains C3 and C4 of *M. mallei* and strain W294 of *Malleomyces pseudomallei* proved to be highly virulent. Animals receiving 30 or more organisms of these strains invariably succumbed to infection, death occurred within 14 days, and usually within 3 to 10 days, depending upon the size of the inoculum. Inoculation of approximately 10 organisms produced a 25 to 35 per cent mortality. Since the MLD was definite and easily determined, it was used in preference to the LD<sub>50</sub>.



Although the virulence of the *M malleri* strains decreased after 1 to 3 months of cultivation on artificial media, it was quickly and easily restored to the original level by 1 or 2 passages through hamsters. Twenty serial passages in hamsters produced no evidence that continued passage would enhance or alter the original virulence of these strains, nor was there any change in the type of disease produced.

Strain C7 was received just before the experimental work was terminated, and only one titration was performed. Injection of 26 organisms (and all larger doses) gave 100 per cent mortality in an average of about 80 hours, and the injection of a calculated dosage of 2.6 organisms resulted in 80 per cent mortality. This strain, recently isolated from a fatal human case, was the most virulent of the *M malleri* strains studied.

*M malleri* strains 2MP, 3MP, and 3PP had a low degree of virulence when received. Large doses produced subacute or chronic infections that resulted in about 80 per cent mortality over a period of 2 to 3 months. The majority of the surviving animals showed evidence of infection (Straus reaction or symptoms of general illness) at some time after inoculation, but recovered spontaneously. A few animals showed no evidence of infection at any time. The virulence of strain 2MP was increased by 4 serial passages by the pulmonary route. The MLD of this passage strain was about 7,000 organisms.

*M malleri* strains C5 and C6 and *M pseudomalleri* strain W295 also proved to be of low virulence and were not used further in animal studies.

#### INFECTIVITY BY VARIOUS PORTALS OF ENTRY

In view of the high infectivity of *M malleri* and *M pseudomalleri* for the hamster by the intraperitoneal route, it appeared of interest to compare the infectivity by this route with that by others.

Titration for infectivity by the subcutaneous route were performed according to the procedure previously described for intraperitoneal titrations.

Infectivity by the respiratory route was determined by producing infectious aerosols in a specially designed apparatus (Rosebury *et al*, 1948). Groups of hamsters were exposed for varying periods of time to aerosols containing different concentrations of organisms in order to give graded doses of the inocula. Hamsters of uniform size were used, and the average volume of air inspired per minute under the experimental conditions employed was determined prior to the test runs (Rosebury *et al*, 1948). The number of organisms inspired (inoculum) was computed from the total volume of aerosol inspired and the determined number of viable organisms per unit volume of aerosol. A total of 149 hamsters were exposed to aerosols of *M malleri* and 256 hamsters to aerosols of *M pseudomalleri*.

The results in table 2 show that the LD<sub>50</sub> (average of several titrations) by the subcutaneous route is the same, within experimental error, as that by the intraperitoneal route. The results of repeated virulence titrations by the respiratory route were somewhat variable. The figures in the table represent the average LD<sub>50</sub> computed from all of the separate titrations. Infectivity by the respiratory route, although somewhat lower than that by the subcutaneous and intraperitoneal routes, was comparatively high.

Attempts were made to ascertain infectivity by the oral route by forced manual feeding of decimal dilutions of the organisms in broth. The morbidity and mortality after such inoculation of either species of *Malleomyces* were very irregular, and no accurate LD<sub>50</sub> dosage could be determined. A dosage of 1 to 5 million organisms infected only 20 to 40 per cent of the animals, whereas doses of 400 to 500 organisms infected a similar percentage. Moreover, in several groups that were given doses between these two extremes, none of the animals became infected.

#### SUSCEPTIBILITY OF VARIOUS ANIMAL SPECIES TO INFECTION

Hamsters, guinea pigs, ferrets, rabbits, black mice, white mice, white rats, and monkeys were tested to determine their susceptibility to infection with *M. mallei* and *M. pseudomallei*.

**Guinea pigs** It has been pointed out by several authors that when guinea pigs are injected with unknown biological materials for the isolation of *M. mallei*, several animals should be injected with each sample since some individuals are much more susceptible than others. On the other hand, the guinea pig has been

TABLE 2  
*Infectivity by various portals of entry (hamsters)*

ORGANISM	NUMBERS OF ORGANISMS IN ONE LD <sub>50</sub>		
	Intraperitoneal	Subcutaneous	Respiratory
<i>M. mallei</i> C3 and C4	12	15	100
<i>M. pseudomallei</i> W294	6	10	70

reported to be uniformly and highly susceptible to infection with *M. pseudomallei* (Stanton and Fletcher, 1932).

Preliminary observations with both species of *Malleomyces* in this laboratory indicated that there was marked individual variation in susceptibility to infection. An evaluation of guinea pig susceptibility to the virulent strains of *Malleomyces* available was, therefore, attempted. This was done by (1) determining the morbidity and mortality following a single large intraperitoneal dose of organisms, and (2) by injecting graded doses intraperitoneally in virulence titrations.

A large group of adult guinea pigs was given an intraperitoneal inoculation of 690,000 organisms of *M. mallei* (strain C4), and another like group was given a similar inoculation of 460,000 organisms of *M. pseudomallei* (strain W294). The day of death was recorded and autopsies were performed. Survivors were killed from 95 to 168 days after inoculation and autopsied. The results are shown in table 3. It will be noted that the results with the two species of *Malleomyces* were closely parallel. Some of the animals in each group died of acute glanders or melioidosis within 15 days, others died of subacute or chronic forms of the diseases after 20 to 100 days. Some of the survivors at autopsy were found to have an active chronic infection after 168 days, but others showed no evidence of active infection or of healed lesions.

A virulence titration was performed in guinea pigs with *M. mallei*, strain C7. This was the most virulent strain of *M. mallei*. The results are shown in table 4. All animals that died had the acute or subacute form of the disease. One or more animals in each group either recovered or showed no evidence of having been infected. Of the survivors only one was found to have foci of active infection at autopsy. It is evident that some individual animals were very resistant to infection. The fact that 2 of 3 animals given 26 organisms died of the disease shows that some individual animals were also very susceptible to infection with *M. mallei*. The calculated LD<sub>50</sub> from this titration was 512 organisms. It is apparent that the chronic form of the disease did not develop with

TABLE 3

*Results of intraperitoneal injection of a single large dose of M. mallei or M. pseudomallei in guinea pigs*

ORGANISM	NO OF ORGANISMS INJECTED (IP)	MORTALITY RATIO	SURVIVORS		PER CENT DEAD OF ACUTE DISEASE	PER CENT DEAD OF SUBACUTE OR CHRONIC DISEASE	PER CENT INFECTED WHEN KILLED	PER CENT RECOVERED OR NO EVIDENCE OF INFECTION
			No	Killed after				
<i>M. mallei</i> C4	690,000	34/46	11	168 days	41	33	6	20
			1	95 days				
<i>M. pseudomallei</i> W294	460,000	27/32	4	168 days	34	49	3	13
			1	125 days				

TABLE 4

*Results of a virulence titration of M. mallei, strain C7, in guinea pigs*

NO OF ORGANISMS INJECTED (IP)	MORTALITY RATIO	AVERAGE DAY OF DEATH	SURVIVORS		NO STILL INFECTED	NO RECOVERED OR NO EVIDENCE OF INFECTION
			No	Killed after		
2,600,000	3/4	17	1	70 days	1	0
260,000	3/4	17	1	63 days	0	1
26,000	2/4	26	2	63 days	0	2
2,600	3/4	12	1	63 days	0	1
260	3/4	15	1	63 days	0	1
26	2/3	23	1	63 days	0	1

this highly virulent strain, as it did after the injection of a single large dose of strain C4. All except one of the animals either developed a fatal acute form of the disease or showed no evidence of infection.

A virulence titration was performed, using *M. pseudomallei*, strain W294, in graded doses intraperitoneally. The dosage given and the results are shown in table 5. Regardless of the size of the dosage, some animals died of the acute disease, some died of the chronic disease, and some either recovered or were never infected. The results of this titration parallel those after a single large dose. The calculated LD<sub>50</sub> from this titration was 440 organisms.

*Ferrets.* Twelve ferrets were used to test the susceptibility of this species to

glanders and melioidosis Four animals were given moderate doses intraperitoneally and the remaining 8 were given graded doses subcutaneously All animals died of acute glanders or melioidosis between 8 and 15 days after inoculation The smallest dosages given were 91 and 73 organisms of *M. mallei* and *M. pseudomallei*, respectively It appears that the ferret is quite susceptible to both diseases and that the MLD values are less than 91 and 73 organisms for *M. mallei* and *M. pseudomallei*, respectively

**Rabbits** Intravenous injection of as high as 300 million organisms of *M. mallei*, strain C4, did not infect rabbits This species is apparently resistant to glanders Rabbits given large doses of *M. pseudomallei*, strain W294, subcutaneously or intravenously developed acute fatal melioidosis The degree of susceptibility of the species to this agent was not evaluated

**Mice** The susceptibility of black mice and white mice to virulent strains of *M. mallei* and *M. pseudomallei* was tested by the inoculation of graded doses of

TABLE 5

Results of a virulence titration of *M. pseudomallei*, strain W294, in guinea pigs

NO OF ORGANISMS IN- JECTED (IP)	MORTALITY RATIO	DEAD OF ACUTE DISEASE		DEAD OF SUBACUTE OR CHRONIC DISEASE		NO OF SURVIVORS*
		No	Average day of death	No	Average day of death	
4,400,000	4/5	2	8	2	22	1
440,000	5/5	1	10	4	45	0
44,000	5/5	2	11	3	46	0
4,400	4/5	1	13	3	31	1
440	3/5	1	10	2	37	2
44	1/4	0		1	26	3

\* Through an oversight these animals were not autopsied when killed 2 months after inoculation

the organisms The results were somewhat irregular Sixty to 75 per cent of the animals developed fatal acute or subacute forms of the diseases following intraperitoneal inoculations of 25 to 30 million organisms Inoculation of less than 450,000 organisms produced no mortality, but a few animals killed 2 to 3 months after inoculation were found to have chronic foci of infection Mice appeared to be relatively resistant to infection with *Malleomyces* organisms

**White rats** Thirty-five adult white rats were used to estimate the susceptibility of this species to glanders and melioidosis Intraperitoneal inoculations of 1 million organisms of *M. mallei*, strain C4, failed to produce any demonstrable infection Intraperitoneal doses of 30 million to 1 billion organisms of *M. pseudomallei*, strain W294, produced a subacute or chronic infection with 30 to 50 per cent mortality Survivors showed no evidence of infection after inoculation or when killed and autopsied after 2 to 3 months Smaller inocula produced no obvious evidence of disease, but foci of infection were found in an occasional animal killed for examination White rats appear to be resistant to infection

with *M. mallei* and only slightly susceptible to infection with the only virulent strain of *M. pseudomallei* studied

**Monkeys** Six *Macaca mulata*, weighing 5 to 7 pounds, were tested for susceptibility by giving them graded doses of virulent *M. mallei* (strain C4) and *M. pseudomallei* (strain W294) subcutaneously. The animals were kept under close observation for 2 months, then they were killed with nembutal and carefully autopsied. The 2 animals given the largest doses (1.5 million organisms) of the 2 organisms developed subcutaneous abscesses at the site of inoculation 4 days after injection. In the monkey infected with *M. mallei*, the abscess was about 2 cm in diameter, drained spontaneously after 4 days, and healed completely after 3 weeks. During this time there was a daily temperature elevation of 1 to 3 degrees, a rapid sedimentation rate, and a marked increase in the white blood cell count with a moderate relative lymphocytosis. There was a loss of about 1 pound in body weight and the animal appeared moderately ill. In the monkey infected with *M. pseudomallei*, the abscess was about the same size,

TABLE 6

*Comparative susceptibility of various species of laboratory animals to experimental glanders and melioidosis*

SPECIES	GLANDERS		MELIOIDOSIS	
	Susceptibility	LD <sub>50</sub> (no. of organisms strains C3 and C4)	Susceptibility	LD <sub>50</sub> (no. of organisms strain W294)
Hamsters	Marked	12	Marked	6
Ferrets	Marked	<90	Marked	<73
Guinea pigs	Moderate	512	Moderate	440
Rabbits	Resistant		Moderate	Not determined
Mice	Slight		Slight	
White rats	Resistant		Slight	
Monkeys	Slight		Slight	

drained spontaneously in 4 days, and healed completely in 2 weeks. The temperature elevation, rapid sedimentation rate, and white blood cell elevation with relative lymphocytosis diminished to normal as soon as the abscess drained.

Specific agglutination and complement fixation tests became positive in both animals within 8 to 14 days after inoculation and the titers rose progressively to a maximum 4 weeks later. Agglutinin titers reached 1:2,560 in the glandered monkey and 1:1,280 in the monkey with melioidosis. The complement-fixing titer reached 1:640 in both animals. Intradermal skin tests using a 1:10 dilution of commercial mallein were negative in both animals 4 and 6 weeks after inoculation.

Both animals regained their normal health and vigor after the abscesses healed and appeared completely normal when killed 2 months after inoculation. Autopsy showed complete healing of the skin lesions and no evidence of specific pathological change elsewhere. Cultures of all organs were negative for *M. mallei* and *M. pseudomallei*.

The 4 animals receiving smaller inocula showed no clinical or laboratory evidence of infection at any time and pathological and cultural examinations at autopsy were negative. It is apparent that rhesus monkeys were only slightly susceptible to subcutaneous inoculation with the virulent strains of *Malleomyces* studied.

**Hamsters** The average MLD values for virulent cultures of *M. mallei* and *M. pseudomallei* were found (see comparative virulence of strains) to be approximately 20 and 15 organisms, respectively, by the intraperitoneal or subcutaneous routes. Because of this high susceptibility, together with the fact that hamsters are easily obtained and simple to care for in large numbers, this species was chosen as the most suitable animal for experimental studies. Although adult male hamsters were sometimes preferred because of the development of the easily recognized Straus reaction, comparative studies showed that they were no more susceptible than were adult females.

#### COMPARATIVE SUSCEPTIBILITY OF THE VARIOUS SPECIES

The results of the studies in the preceding sections of this paper are summarized in table 6 to facilitate comparisons.

#### DISCUSSION

*M. mallei* strains 2MP, 3MP, and 3PP were of very low virulence and produced subacute or chronic infection in hamsters. The moderately virulent strains C3 and C4 produced acute fulminating infections in hamsters and ferrets but only subacute or chronic infections in the majority of guinea pigs. The highly virulent strain C7 produced acute fulminating forms of the disease in both hamsters and guinea pigs. It appears that the virulence of the strain has a great deal to do with the type of disease that is produced. This is undoubtedly a partial explanation for the occurrence in horses of all gradations of the disease from acute fulminating glanders to the chronic low-grade form of farcy. It may also serve to explain why the disease never became widespread in man, but occasionally in the presence of outbreaks of fulminating infections in horses a large number of human cases developed.

The two strains of *M. pseudomallei* were reported to be avirulent stock laboratory cultures. Strain W295 proved to be avirulent for experimental animals, but strain W294 appeared to be highly virulent.

The relatively low MLD values in hamsters by subcutaneous, intraperitoneal, and respiratory routes emphasized the high degree of infectivity of virulent *Malleomyces* organisms in a susceptible host. The erratic results after oral inoculation suggests that these organisms are often readily destroyed after ingestion. However, the fact that a small oral inoculum produced infection in 20 to 40 per cent of the animals indicated that this may be an important route of natural infection.

The uniformly high degree of susceptibility of hamsters to glanders and melioidosis compared with the irregular susceptibility of guinea pigs emphasizes the value of hamsters for experimental work. The irregular infectivity of *M.*

*pseudomallei* (strain W294) for guinea pigs contrasts with the uniform infectivity reported by Stanton and Fletcher (1932). Since their strains were freshly isolated from cases of the natural disease, this discrepancy may be due entirely to a difference in virulence of strains. The low degree of susceptibility of rats to melioidosis and the development of chronic forms of the disease emphasizes the role this species may play as a natural reservoir of infection.

#### SUMMARY

The various strains of *Malleomyces mallei* and *Malleomyces pseudomallei* studied varied greatly in their virulence. The strains of low virulence tended to produce subacute or chronic forms of the diseases, whereas strains of high virulence produced acute fulminating infections. The virulence of one strain (2MP) was increased by serial passage in hamsters.

Hamsters were easily infected by inoculation by the intraperitoneal, subcutaneous, and respiratory routes. Oral inoculation gave irregular results, but some animals became infected after the ingestion of relatively small doses.

Of the laboratory animals tested hamsters were found to be the most susceptible to both diseases. Ferrets were also very susceptible, but guinea pigs were only moderately susceptible and individual animals varied a great deal in the degree of susceptibility. Rabbits, mice, rats, and monkeys were least susceptible.

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## NOTE

### CHANGES INDUCED IN THE 1,2,3 ANTIGENS OF SALMONELLA<sup>1</sup>

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In a previous note on the nonspecific phases of *Salmonella* (J Bact , 53, 359) it was stated that *S newport* (VI, VIII e, h-1,2,3) had been changed to a form indistinguishable from *S pueris* (VI, VIII e, h-1,2), and the 1,2,3 phases of *S oregon* and *S vejle* were changed to 1,2. Since then the 1,2,3 phases of *S typhi-murium*, *S heidelberg*, *S saint-paul*, *S coeln*, *S virchow*, *S concord*, *S richmond*, *S litchfield*, *S amager*, *S aberdeen*, *S pretoria*, *S szentes*, and *S fayed* were changed to 1,2 by the method previously described. This is not simply a loss variation since 1,2 phases contain antigens not found in 1,2,3 phases and the change involves a gain as well as a loss in antigen. In *S litchfield* the 1,2,3 phase was changed to 1,2 and then reversed to 1,2,3.

In *S stanley* (IV, V, XII d-1,2) the naturally occurring 1,2 phase was transformed to 1,2,3 and then changed back to 1,2. The other natural 1,2 phases of the genus proved resistant to change. In all the transformations mentioned above it should be emphasized that the results were supported by absorption tests as well as by agglutination with single factor serums.

It was possible to prepare a very strong 3 serum by absorption of high-titered serum for phase 2 of *S newport* by very dense suspensions of *S pueris*. This serum, when used in low dilution, agglutinated not only 1,2,3 phases but also acted upon 1,7 phases and many of the 1,5 phases. Identical results were obtained when the serum was absorbed with phase 2 of *S paratyphi B*.

Since antigen 3 is present in small amounts in so many of the nonspecific phases, and since it can be made to appear and disappear by manipulation, it is suggested that the antigen be omitted from the Kauffmann-White schema. This will result in a slight reduction of the number of types and will not affect the epidemiological value of the schema. It will also obviate the necessity of single factor 3 serum, a reagent that is rather difficult to prepare.

<sup>1</sup> The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station, Lexington, and is published by permission of the Director. It was supported in part by a research grant from the U S Public Health Service.



# INDUCED RESISTANCE OF STAPHYLOCOCCUS AUREUS TO VARIOUS ANTIBIOTICS

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In the course of studies on the mode of action of various antibiotics, it was observed (Cavallito and Bailey, 1944, Cavallito *et al* , 1945) that a variety of antibacterial substances could be inactivated by various thiol compounds, particularly cysteine. Antibacterial agents may be classified, on the basis of their behavior with thiols, into several groups: some react rapidly with a large variety of —SH compounds, others react rapidly only with cysteine or related  $\beta$ -amino-alkane thiols, and others display reactions intermediate between these two extremes (Cavallito, 1946, Bailey and Cavallito, 1946). This pattern of behavior of certain antibiotic agents toward thiol compounds prompted a study of the development of resistance of *Staphylococcus aureus* to several of the antibiotics to determine whether a correlation existed between development of resistance and reactivity with thiol compounds.

## METHODS

*Staphylococcus aureus* 209P (ATCC 6538P) was used throughout these studies. The antibacterial agents studied were penicillin, streptomycin, pyocyanin, gliotoxin, aspergillic acid, mercuric chloride, and the active principles of *Allium sativum*, *Asarum canadense*, and *Archim minus*. The medium employed was a beef extract broth at pH 7.0. The susceptibility of *S. aureus* to the various antibiotics was determined by growing the organism in a series of broth cultures containing increasing quantities of the antibiotic under test. The total volume of fluid in each tube of the series was 5 ml, the inoculum consisted of 1 ml of a 1:1,000 dilution of an 18-hour culture in similar medium. Stock solutions of the antibiotics were prepared in phosphate buffer pH 7.0 with the exceptions of streptomycin, of which aqueous solutions were used, and aspergillic acid, for which bicarbonate buffer was found to be the most satisfactory solvent. All working dilutions were prepared in beef extract broth pH 7.0.

## EVALUATION OF RESISTANCE

The index of resistance was the lowest concentration of the antibiotic under test that produced complete inhibition of growth after 18 hours of incubation at 37°C. Resistance of the organism to the antibiotic under study was induced by inoculation of the culture, from the tube that contained the maximum amount of antibiotic that had permitted growth, to a fresh series of tubes containing appropriate concentrations of antibiotic. In this manner the culture growing in the largest quantity of antibiotic was transferred serially from day to day. Dilutions of antibiotic were so prepared as to provide latitude for variation on either

side of the concentration of antibiotic in the culture selected as inoculum. This procedure minimized the possibility of losing the resistant organism. Readings for the presence or absence of growth were recorded at the end of 18 to 24 hours. In the first 15 to 20 transfers, subcultures were made daily, thereafter transfers were made at the end of 48 hours. This procedure did not affect the end point observed by more than one step in the dilution series and permitted a more luxuriant growth of the resistant culture.

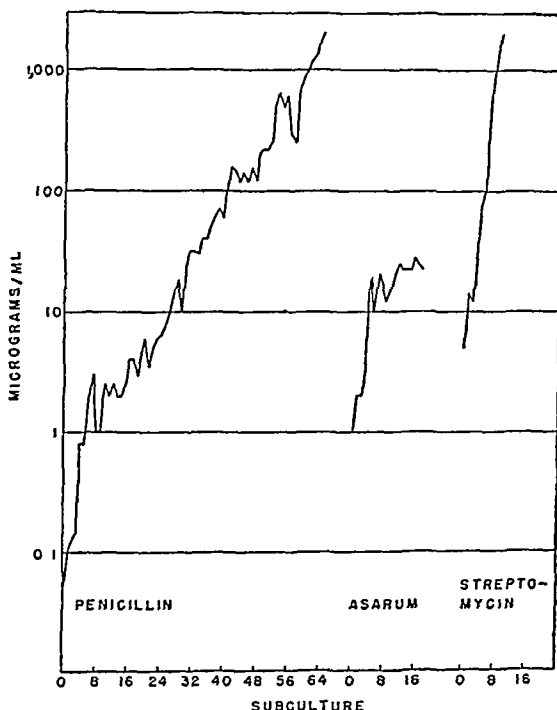


FIG 1 RESISTANCE OF STAPHYLOCOCCUS AUREUS 209 TO ANTIBIOTICS

#### DEVELOPMENT OF RESISTANCE

Patterns of resistance observed for the various antibiotics are illustrated in figures 1, 2, and 3. Under the conditions of the experiment *S. aureus* was inhibited by crystalline triethylamine salt of penicillin G in a concentration of 0.05 micrograms per ml. During successive transfers in the presence of penicillin, there was a progressive increase in resistance until, after 64 transfers, the organism grew well in a concentration of 1 mg per ml of penicillin, an increase in resistance of 20,000 times that of the sensitive parent organism. This increase in resistance fluctuated with each serial transfer and illustrates the necessity of using series of dilutions rather than a single concentration at the level at which the resistant culture was expected to grow. The pattern of development of resistance is in keeping with the previously reported observations of Todd, Turner, and Drew (1945).

Continued transfers into increasing concentrations of penicillin resulted in obtaining a strain of *S. aureus* that grew in the presence of 4 mg of penicillin per

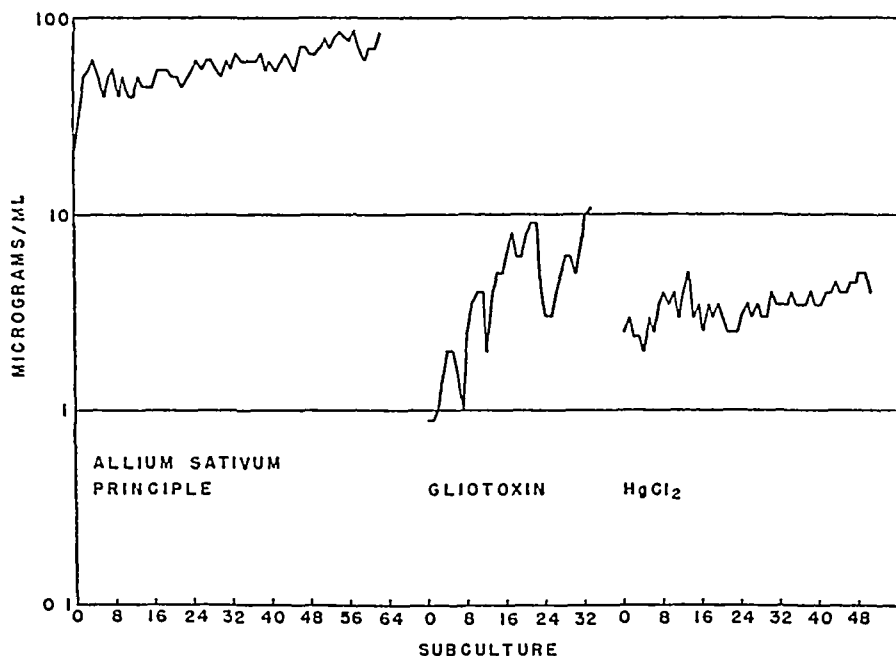


FIG 2 RESISTANCE OF STAPHYLOCOCCUS AUREUS 209 TO ANTIBIOTICS

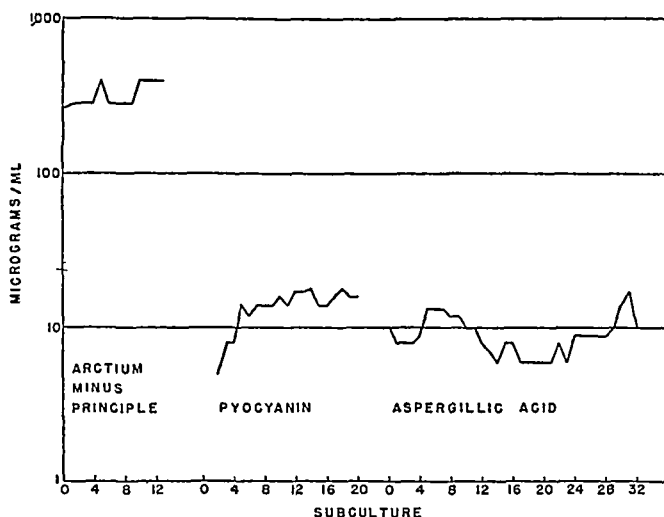


FIG 3 RESISTANCE OF STAPHYLOCOCCUS AUREUS 209 TO ANTIBIOTICS

ml of culture medium—an increase of resistance 80,000 times that of the parent organism. Less fluctuation was noted at resistance levels of 1,000 to 4,000 micrograms. It is also significant that at these high levels of resistance the size

of inoculum was of little importance. We have found that below 1 mg per ml, resistance could be increased more readily, and with less fluctuation in the pattern, by the use of heavy inocula. No attempt was made to increase the resistance of this organism beyond 4 mg per ml.

With the increase in resistance of *S. aureus* to penicillin a gradual decrease was observed in all of the fermentative properties characteristic of the parent strain, at 1 mg per ml level all of the fermentation reactions were almost completely suppressed. Pigmentation was first reduced and then was completely lost, hemolysis was retarded and the coagulase reaction was negative in the single test conducted.

Microscopic examination of the resistant cultures was made at intervals, and the enlargement of the cocci, reported by Gardner (1940) and others, was observed up to approximately 1 mg levels. At this level a complete morphologic and tinctorial change was observed. The culture became highly pleomorphic, showing elements varying from cocci to coccobacilli and occasional diphtheroid forms. All of these elements stained gram-negative. In view of this radical change in the microscopic picture from that usually observed, a subculture was transferred to penicillin-free broth for study (this strain will be discussed below under "Reversal of Resistance"). Another subculture was continued as before in penicillin-containing medium. The stabilization of this strain by repeated culturing in a medium containing 4 mg of penicillin per ml of medium resulted in what appeared to be a fixed type of morphology.

The resistance of *S. aureus* to streptomycin was found to develop at a rapid rate. During the course of 7 daily subculturings in broth containing this antibiotic, the resistance of the culture increased from 0.5 micrograms to 1 mg per ml, after 12 transfers the organism grew well in a broth containing 4 mg per ml of streptomycin. No attempt was made to increase the resistance of the culture beyond this point.

In contrast to the situation with penicillin, no significant morphologic changes were observed in the culture during the course of these experiments with streptomycin. As with penicillin, a retarding effect of streptomycin on the characteristic fermentation reactions of *S. aureus* was observed.

The active principle "A" of *Asarum canadense* (Cavallito and Bailey, 1946) showed approximately the same pattern with respect to the development of resistance by *S. aureus* as did penicillin. The degree to which resistances could be built up was limited by the low aqueous solubility of the active principle.

*Staphylococcus aureus* developed only slight resistance to allyl-2-propene-1-thiolsulfinate (Cavallito, Buck, and Suter, 1944) or to the active principle of *Arctium minus* (Cavallito, Bailey, and Kirchner, 1945, Cavallito and Kirchner, 1947). Prolonged exposure to the former compound resulted in but a slight decrease in the sensitivity of the organism to this antibiotic. Whatever resistance was developed appeared early, and further culturing of the organism in the presence of the antibiotic was without effect. No morphological change was observed in organisms grown for 61 transfers in the presence of this antibiotic. The biochemical reactions of the test organism, however, were suppressed. A similar pattern was observed with the *Arctium minus* principle.

A moderate degree of resistance was developed by *S. aureus* to pyocyanin and ghotoxin after a prolonged series of exposures. The values obtained were intermediate between those obtained for the antibiotics that induced marked and minimal degrees of resistance. Definite changes in biochemical reactions were observed, morphologically the culture did not show any change. No significant resistance to mercuric chloride was developed by *S. aureus* during 50 transfers in the presence of this agent.

Cultures of *S. aureus* exposed to the action of aspergillic acid showed considerable fluctuation in the development of resistance. After 32 serial transfers in a medium containing varying quantities of the antibiotic, no significant degree of resistance was developed.

#### REVERSAL OF RESISTANCE

When the culture had developed resistance to 1 mg of penicillin per ml, a subculture was made in broth containing no penicillin. It will be recalled that this strain had altered biochemical properties, showed marked changes in morphology, and was gram-negative. Subculturing in penicillin-free broth brought about a gradual return to normal in biochemical reactions, morphology, and staining reaction. After the sixth subculture in penicillin-free broth very few coccobacilli or diphtheroid forms could be found. After 10 such subcultures only typical morphology was observed. During the first 10 transfers, the period when the morphology and biochemical reactions were returning to normal, a rapid loss in resistance was observed. Thereafter a more gradual loss in resistance was noted, after 63 transfers this strain was susceptible to 0.1 microgram per ml, a value only twice that of the parent culture.

With the strain of *S. aureus* that was resistant to 4 mg of penicillin per ml and stabilized at that level, a different situation was found, this strain remained resistant during 40 transfers into penicillin-free extract broth. Other methods brought about a return to typical morphology and biochemical reactions as well as a reduced resistance (Bellamy and Klimek, 1948).

With the streptomycin-resistant strain of *S. aureus*, reversal was quite uncertain in nature. End points varied with successive subcultures with no certainty that the same, higher, or lower end points might be attained. Indications were, however, that only a moderate reversal occurred.

Allyl-2-propene-1-thiolsulfinate-resistant and pyocyanin-resistant strains retained their resistance for 48 and 22 subcultures, respectively, whereas ghotoxin behaved in a manner similar to penicillin. No attempt was made to reverse the resistance obtained to the active principles of *Arctium minus* or *A. sarum canadense*.

#### CROSS RESISTANCE WITH PENICILLIN AND STREPTOMYCIN

No cross resistance with streptomycin could be demonstrated with the penicillin-resistant strain at susceptibility levels below 1 mg per ml. When this strain, however, had developed a resistance to more than 1 mg per ml of penicillin, it was resistant to 15 to 20 micrograms of streptomycin. This organism had a normal susceptibility to streptomycin of 0.5 micrograms per ml. Similarly, the

streptomycin-resistant strain was not inhibited by 1 microgram per ml of penicillin, an increase of 20 times the resistance of the parent strain

#### DISCUSSION

From the data presented, it appears that there are two extremes in types of antibiotics relative to the ability of bacteria to develop resistance to them. At the one end is the group that is characterized by rapid development of resistance, with streptomycin, penicillin, and the active principle of *Asarum* serving as examples, and at the other extreme is allyl-2-propene-1-thiolsulfinate and the active principle of *Arctium minus*. Pyocyanin and gliotoxin are intermediate with respect to the development of resistance, indicating that a gradation may exist between the two extremes.

It is interesting to observe that this group of antibacterial agents shows a correlation between the ability to develop resistance and the specificity of reactivity with sulfhydryl groups. Those antibiotics that are more selective as to the type of —SH with which they can react are also more likely to be capable of inducing marked bacterial resistance to their action. The data given by Cavallito (1946), which showed the specificity of reactivity with thiols, may be used for estimating the capacity for the development of resistance.

Aspergillic acid appears to lack the power to stimulate development of resistance by *S. aureus*. This antibiotic failed to react with any of the thiol compounds tested.

It is of further interest to note that nonreversible bacteriostatic systems are formed by those antibiotics that show marked ability to induce resistance and that also react selectively with thiol compounds (Bailey and Cavallito, 1946). Reversible bacteriostatic systems are formed by those antibiotics that react nonselectively with thiols and to which little resistance can be developed.

#### SUMMARY

*Staphylococcus aureus* 209P (ATCC 6538P) can develop marked resistance to penicillin, streptomycin, and the active principle "A" of *Asarum canadense*. This organism developed an intermediate degree of resistance to both pyocyanin and gliotoxin, whereas very little resistance was developed to allyl-2-propene-1-thiolsulfinate, mercuric chloride, or to the active principle of *Arctium minus*. This organism failed, under the conditions of these experiments, to develop resistance to aspergillic acid.

*S. aureus* has been rendered resistant to a concentration of penicillin of 4 mg per ml. At a concentration of 1 mg per ml the characteristic biochemical reactions of *S. aureus* were lost, and the organism became markedly pleomorphic and gram-negative. The resistance of the strain was apparently stabilized by repeated transfers in broth containing 4 mg per ml of penicillin.

The biochemical, tinctorial, and morphological characteristics of the parent culture were regained when the strain that was resistant to 1 mg per ml of penicillin was cultured in penicillin-free medium.

During 12 transfers in increasing concentrations of streptomycin, *S. aureus* developed a resistance to 4 mg per ml of that antibiotic.



All of the characteristic biochemical reactions of the organism were suppressed at this level of resistance, but no noticeable change in morphology was observed

At high levels of resistance to penicillin and streptomycin slight cross resistance to these antibiotics could be demonstrated

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# THE RELATION BETWEEN INDUCED RESISTANCE TO PENICILLIN AND OXYGEN UTILIZATION

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A search of the literature reveals many cases of artificially induced resistance to antibiotics. Abraham *et al* (1941) developed a variant of *Staphylococcus aureus* one thousand times more resistant to penicillin than the parent strain. Numerous authors (Gardner, 1940, Smith and Hay, 1942, Fisher, 1946, and Klimek *et al*, 1948) have reported that growth in the presence of penicillin may be accompanied by such morphological changes as pleomorphism, filament formation, enlarged cells, and loss of the gram-positive character. There have been few reports, however, on the physiological changes caused by exposure to penicillin. Lyons (1943) has reported that penicillin-resistant staphylococci were unaltered in coagulase activity and in the fermentation of mannitol. No loss of virulence was observed. Abraham and Chain (1940) reported that induced resistance of staphylococci to penicillin is not accompanied by the production of a penicillin-destroying enzyme.

Since the mode of action of antibiotics may be determined by physiological and metabolic studies, rather than by morphological studies, the present work was undertaken to determine more completely the physiological changes brought about by induced resistance to penicillin.

Both Klimek *et al* (1948) and Abraham *et al* (1941) reported that penicillin-resistant staphylococci grow more slowly than did the parent strain. Highly resistant variants were observed to grow almost exclusively at the surface of broth cultures. It was this observation which led the authors to suggest the loss of anaerobiosis as a possible cause for slower growth of the penicillin-resistant variant. A corollary of this theory would suggest that organisms that cannot grow aerobically would develop little resistance to penicillin. This might explain the inability of McKee and Hauck (1943) to obtain greater than 30-fold increase in the resistance of streptococci and pneumococci as compared to an increase of 1,000-fold to 6,000-fold for staphylococci.

The data presented in this paper show that a penicillin-resistant variant of *S. aureus* has lost the ability to grow anaerobically. Under similar experimental conditions certain other organisms that cannot grow aerobically developed little resistance to penicillin.

## CULTURES AND METHODS

Klimek *et al* (1948) developed P<sub>60</sub>T<sub>24</sub>, a penicillin-resistant variant of *Staphylococcus aureus* 209P, which grows readily in the presence of 4 mg per ml of penicillin. This variant was obtained by serial transfer in broth containing increasing concentrations of penicillin. Variant P<sub>60</sub>T<sub>24</sub>, the parent strain *S*

*aureus* FDA 209P (American Type Culture Collection 6538P), *Streptococcus faecalis* 10Cl, and *Streptococcus mastitidis* 68C from the Laboratory of Bacteriology at Cornell University, and *Clostridium welchii* M from this laboratory were used throughout these experiments

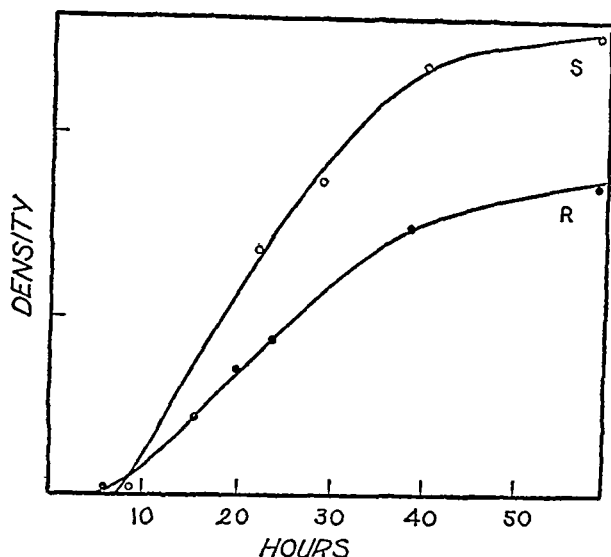


FIG 1 AEROBIC GROWTH OF PENICILLIN-SENSITIVE S AUREUS 209 P (S) AND PENICILLIN-RESISTANT P<sub>60</sub> T<sub>34</sub> (R)

"Optical density" determined with Evelyn colorimeter at 660 mμ

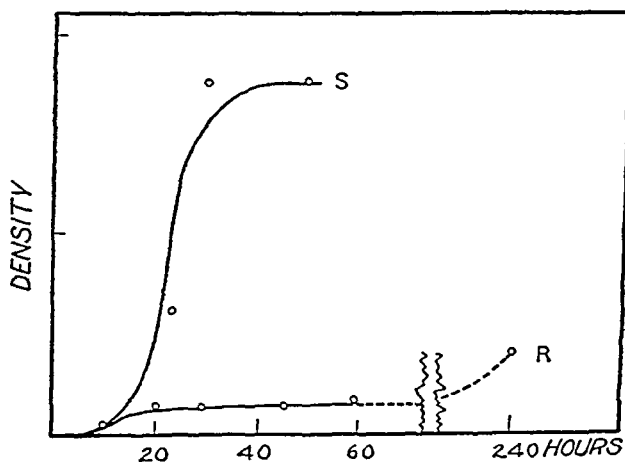


FIG 2 ANAEROBIC GROWTH OF PENICILLIN-SENSITIVE S AUREUS 209 P (S) AND PENICILLIN-RESISTANT P<sub>60</sub> T<sub>34</sub> (R)

"Optical density" determined with Evelyn colorimeter at 660 mμ

Aerobic conditions were obtained by growing the culture in 10 ml of broth in 250-ml Erlenmeyer flasks. Anaerobic growth was determined in broth under a "vaspar" seal. The amount of growth was determined turbidimetrically using an Evelyn colorimeter with the 660 millimicron filter. The streptococci were

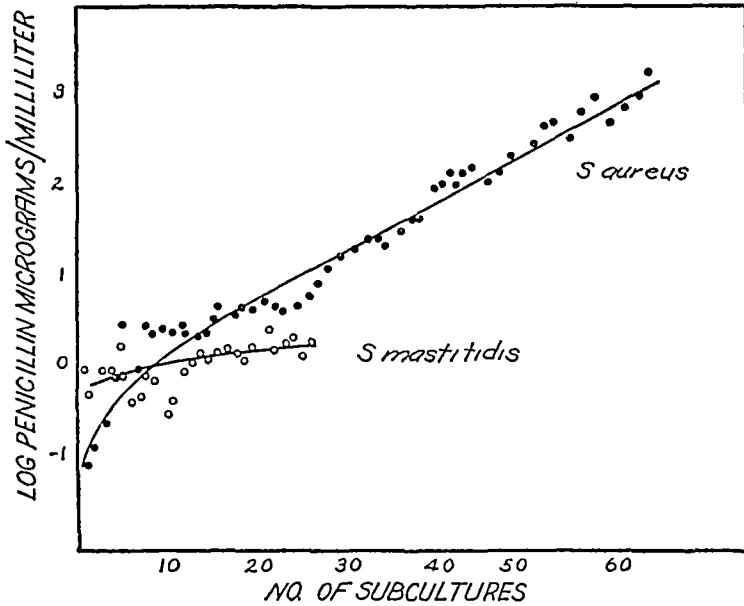


FIG 3 DEVELOPMENT OF INDUCED RESISTANCE BY SERIAL TRANSFER IN PENICILLIN-CONTAINING BROTH

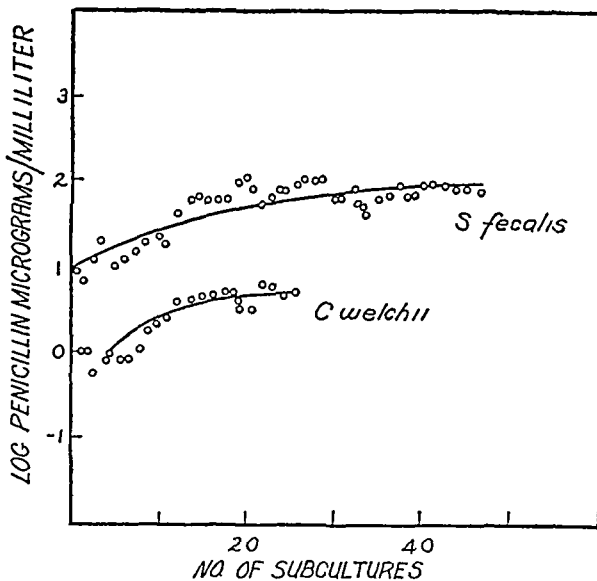


FIG 4 DEVELOPMENT OF INDUCED RESISTANCE BY SERIAL TRANSFER IN PENICILLIN-CONTAINING BROTH

grown in a medium consisting of Difco yeast extract, 1 per cent, Sheffield "N-Z amine," 1 per cent, dipotassium phosphate, 0.5 per cent, and glucose, 0.1 per cent. The clostridium was grown in Difco anaerobe medium, which does not

contain any penicillin-inactivating thioglycolate. The medium was boiled immediately before use to expel oxygen, then cooled, and the penicillin added immediately preceding inoculation. Crystalline penicillin G was used in all experiments.

#### RESULTS

Figures 1 and 2 represent aerobic and anaerobic growth curves of *S. aureus* 209P and P<sub>60</sub>T<sub>24</sub>. Aerobically, the resistant variant grew about one-half as fast and reached about eight-tenths the final turbidity of the sensitive, parent culture. Anaerobically, P<sub>60</sub>T<sub>24</sub> did not grow at an appreciable rate.

The results of attempts to induce penicillin resistance in the other three organisms by the serial dilution method are presented in figures 3 and 4. The resistance curve for *S. aureus* 209 (obtained by Klimek *et al.*, 1948) is included for comparison. The resistance of *S. faecalis* 10Cl increased 11 times in 47 transfers, that of *S. mastitidis* 68C 6 times in 24 transfers, and that of *C. welchii* M 10 times in 25 transfers.

#### DISCUSSION

The evidence strongly suggests that penicillin interferes with one or more essential components in the anaerobic energy mechanism of susceptible organisms. Although several authors (Burke, 1939, Barron and Friedeman, 1941) studied pathways for carbohydrate utilization other than the Meyerhoff-Emden scheme, little is known about the energy relations involved. Intermediates have been neither isolated nor characterized. Therefore, the problem of determining the exact site of inhibition is complex.

Triose phosphate dehydrogenase, the enzyme responsible for the oxidation of 1,3-diphosphoglyceraldehyde to 1,3-diphosphoglyceric acid, is known to be inhibited by iodoacetate or iodoacetamide. It was thought that the use of these inhibitors could indicate whether the resistant variant utilized this enzyme. It was found, however, that *S. aureus* 209 and P<sub>60</sub>T<sub>24</sub> are equally sensitive to iodoacetamide, thus suggesting that both variants utilize triose phosphate dehydrogenase.

#### SUMMARY

A penicillin-resistant variant of *Staphylococcus aureus* has been obtained which grows more slowly than the sensitive, parent culture. This resistant variant has lost the ability to grow anaerobically. Strains of *Streptococcus faecalis*, *Streptococcus mastitidis*, and *Clostridium welchii* when treated in a similar manner failed to develop significant resistance to penicillin. From these data it is suggested that organisms that depend upon anaerobic processes for their energy supply will not develop appreciable resistance to penicillin.

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# SOME PROPERTIES OF PENICILLIN-RESISTANT STAPHYLOCOCCI

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Since Fleming's discovery of penicillin many studies have been undertaken to determine its mode of action. The result has been a plethora of dialectic speculation and a paucity of facts. Penicillin is primarily effective against gram-positive bacteria. Chan and Duthie (1945) observed that penicillin inhibits the respiration of young growing staphylococci, whereas the respiration of resting cells is unimpaired. Krampitz and Werkman (1947) reported that penicillin inhibits the endogenous oxygen uptake and the oxidation of ribonucleotides, whereas the oxidation of glucose was unimpaired. Gale and Taylor (1946) found that penicillin prevents the assimilation of glutamic acid by staphylococci. This loss precedes the loss of respiration and lysis and appears to be coincident with the loss of viability.

The great amount of time, energy, and money spent on the study of the mechanism of penicillin action indicates the general agreement that more intelligent penicillin therapy as well as a more logical approach to the synthesis of penicillinlike compounds could result from a more complete knowledge of the mode of action. The authors have chosen to study the problem by a comparison of the properties of penicillin-resistant variants with those of the parent sensitive culture. Klimek *et al* (1948) reported the development of a variant of *Staphylococcus aureus* which was 60,000 times more resistant to penicillin than was the parent culture. The resistant variant was found to be gram-negative. The authors found that this variant had lost the ability to grow anaerobically. This paper reports further studies of the properties of the penicillin-resistant variant which indicate a progressive loss of fermentative activity, the gain of the ability to synthesize nicotinic acid, the production of an extracellular penicillinase, and a method for reversing the penicillin-resistant gram-negative variant to a gram-positive coccus.

## MATERIAL AND METHODS

The cultures used throughout these experiments were *Staphylococcus aureus* FDA 209P (American Type Culture Collection 6538P), penicillin-resistant variants of *S. aureus* 209P developed by Klimek *et al* (1948), and *Escherichia coli* 4157 and *Bacillus subtilis* S<sub>8</sub> from the Laboratory of Bacteriology at Cornell University. Crystalline sodium penicillin G and the usual bacteriological techniques were employed. The increased resistance to penicillin of variants of *S. aureus* 209P is indicated in parentheses after the number of the variant, e.g., P<sub>28</sub> (250 ×).

## CARBOHYDRATE FERMENTATION

The production of acid from certain carbohydrates has been considered one of the most constant physiological characteristics of staphylococci. Chapman (1946) and Blair (1938) reported that the most reliable of these carbohydrates are glucose, sucrose, lactose, maltose, and mannitol.

Fermentation tests were run from time to time on certain resistant variants. Originally phenol red broth was used as the base for the determination of acid production from carbohydrates, but when it was found (Bellamy and Klimek, 1948) that the more resistant variants grew only aerobically, phenol red agar base was used. Table 1 illustrates the results of acid production in phenol red broth. The observations were complicated by the slower rate of growth of the resistant variants, in some instances a week or more was required for the production of a visible acid reaction.

TABLE 1

*Fermentation by penicillin-resistant variants of S aureus 209 using phenol red broth*

VARIANT	GLUCOSE	SUCROSE	LACTOSE	GALACTOSE	MALTOSE	MANNITOL
209*	+	+	+	+	+	+
P <sub>28</sub> (250 ×)	+			+	—	+
P <sub>34</sub> (1,080 ×)	+			+	—	sl
P <sub>54</sub> (10,000 ×)	sl		sl	—	—	—

\* All cultures were derived from the original penicillin-sensitive *S aureus* 209P

Pa where a = number of subcultures in increasing concentrations of penicillin

Pb Tb where b = number of transfers at the highest concentration of penicillin

Pa Tb Rc where c = number of transfers in the absence of penicillin, i.e., the reversal transfers

Table 2 presents the results obtained with phenol red agar. Although reduction of nitrates and growth in 6.5 per cent sodium chloride are not fermentation reactions, they are included in this table because of their frequent use as biochemical characteristics of *S aureus* (Chapman, 1945).

Increase in resistance was accompanied by a progressive loss in physiological functions. Of the properties listed only the production of acid from glucose remained.

## NUTRITIONAL REQUIREMENTS

The nutritional requirements of *S aureus* have been investigated by Fildes and Richardson (1937), Gladstone (1937), and Knight (1937). A mercapto group (—SH), either actual or potential, is necessary as a sulfur source. Nicotinic acid or nicotinamide and thiamine are the only growth factors required. Freshly isolated strains require several amino acids for growth, but training after the manner of Knight (1936) will produce strains capable of utilizing ammonia as the sole source of nitrogen.

Table 3 presents the results of a typical experiment on the comparison of the

growth factor requirement of the parent strain with that of the highly resistant variant  $P_{60}T_{39}$  (60,000  $\times$ )

TABLE 2

*Fermentation by penicillin-resistant variants of S aureus 209P using phenol red agar*

VARIANT	GLUCOSE	SUCROSE	LACTOSE	MANNITOL	NITRATE REDUCTION	6.5% NaCl
209P*	+	+	+	+	+	+
$P_{12}T_4$ (1,000 $\times$ )	+	+	+	+	+	—
$P_{46}$ (4,000 $\times$ )	+	—	+	—	+	—
$P_{60}T_{41}$ (60,000 $\times$ )	+	—	—	—	—	—
$P_{77}T_3$ (60,000 $\times$ )	+	—	—	—	—	—
$P_{60}T_{28}R_{21}$ (60,000 $\times$ )	+	—	—	—	—	—
$P_{77}T_2R_4$ (60,000 $\times$ )	+	—	—	—	—	—

\* See table 1

TABLE 3

*The effect of nicotinic acid and thiamine on the growth of variants of S aureus 209P*

BASE MEDIUM* 10 ML	THIAMINE 100 $\mu$ G	NICOTINIC ACID 100 $\mu$ G	GROWTH† 48 HOURS
209P +	+	+	0 127
+	+	+	0 127
+	+	—	0 051
+	+	—	0 051
+	—	+	0 030
+	—	+	0 022
+	—	—	0 017
+	—	—	0 013
$P_{60}T_{39}$ +	+	+	0 269
+	+	+	0 236
+	+	—	0 242
+	+	—	0 242
+	—	+	0 022
+	—	+	0 022
+	—	—	0 045
+	—	—	0 058

\* Base 25 ml 10% vitamin free acid-hydrolyzed casein, 5 ml "salts B," 7 ml  $M/2$   $K_2HPO_4$ , 200 mg cystine, water to 1,000 ml

"Salts B"  $MgSO_4 \cdot 7H_2O$ , 10 g, NaCl, 0.5 g,  $FeSO_4 \cdot 7H_2O$ , 0.5 g,  $MnSO_4 \cdot 4H_2O$ , 0.5 g, water, 250 ml

† Density as determined by Evelyn colorimeter  $\lambda = 660 m\mu$

The growth of  $P_{60}T_{39}$  in the absence of nicotinamide was wholly unexpected, but this observation has been confirmed several times. Since  $P_{60}T_{39}$  grew in the absence of nicotinic acid, there remained the possibility of synthesis of nicotinamide by this variant. The question was settled by assaying the medium after growth. The nicotinic-acid-free medium used for the growth of the

cultures is the base medium described in the footnote to table 4. Penicillin-resistant  $P_{60}T_{42}$  was grown aerobically in the medium at 37 C for 48 hours. Twenty ml of these cells and medium were autoclaved for 15 minutes at 15 pounds' pressure with 1 ml of 2.5 N sodium hydroxide. After neutralization aliquots were removed for assay. Table 4 represents the growth of parent penicillin-sensitive *S. aureus* 209 using the foregoing source of nicotinic acid. The growth was estimated by titration of the acid formed. It is apparent that

TABLE 4

*Growth of S. aureus 209P using  $P_{60}T_{42}$  as the source of nicotinamide*

SOURCE OF N A			GROWTH (ACID PROTECTION)
			ml N/10 NaOH
Base medium*			0 0
" "			0 0
" "	+ NA 100 $\mu$ g		3 6
" "	" "		2 5
" "	+ $P_{60}T_{42}$		4 7
" "	" "		4 2

\* Base medium: glucose, 0.5%, ammonium chloride, 0.2%, "salts B," 0.2%, M/2  $K_2HPO_4$ , 0.7%, M/2  $KH_2PO_4$ , 0.2%. After autoclaving the following were added aseptically: thioglycolate, 0.01%, arginine, 0.01%, glycine, 0.01%, glutamic acid, 0.01%, uracil, 0.01%, thiamine, 1 mg %.

TABLE 5

*Nicotinic acid assay of medium after growth, using L. arabinosus*

SOURCE OF N A			N A AFTER GROWTH
			$\mu$ g/ml
Base medium*			0 008
" "			0 008
" "	+ NA 100 $\mu$ g		too great to determine
" "	" "		" " " "
" "	+ <i>B. subtilis</i> S <sub>3</sub>		0 372
" "	" "		0 466
" "	+ <i>E. coli</i> 4157		0 058
" "	" "		0 052
" "	+ <i>S. aureus</i> $P_{60}T_{42}$		0 382
" "	" "		0 372

\* Base medium same as in table 4

$P_{60}T_{42}$  has supplied sufficient nicotinic acid for optimum growth. A quantitative assay for nicotinic acid was run using the *Lactobacillus arabinosus* method of USP XIII (1947). *Escherichia coli* 4157 and *Bacillus subtilis* S<sub>3</sub>, two organisms known to synthesize nicotinic acid, were included in the experiment for comparison (table 5).

It can be seen that, under the foregoing conditions,  $P_{60}T_{42}$  synthesized as much nicotinamide as did *B. subtilis* S<sub>3</sub> and several times as much as did *E. coli* 4157.

## PENICILLINASE

Many strains of *Staphylococcus aureus* produce penicillinase (Bondi and Dietz, 1945), but *S. aureus* 209P does not (Luria (1947), Spink and Ferris (1947), and others have reported that the penicillinase produced by staphylococci was intracellular and could not be obtained cell-free.

All attempts to demonstrate an active penicillinase in cells of intermediate resistance were unsuccessful. The following experiments are representative of attempts to demonstrate penicillinase activity of intermediate variants.  $P_{32}$  (1,000  $\times$ ) was grown in quantity and extracted after the manner of Benedict *et al* (1945), but no penicillinase activity was obtained. A sterile filtrate of  $P_{36}$  (1,350  $\times$ ) contained no demonstrable penicillinase. The cells of  $P_{60}$  (12,500  $\times$ ) harvested from one liter of broth containing 1 mg of penicillin per ml were suspended within a collodion sac in sterile broth containing 10  $\mu$ g penicillin per ml. No loss of penicillin could be demonstrated in the surrounding broth.

TABLE 6

*Penicillinase production by  $P_{60}T_{48}$  as indicated by the growth of 209P in the presence of 20  $\mu$ g per ml of penicillin*

MG PER ML PENICILLIN IN GROWTH MEDIUM OF $P_{60}T_{48}$	ML OF FILTRATE ADDED TO 10 ML BROTH*			
	0	0.01	0.1	1.0
0	0	0	0	0
0.2	0	0	0	++
4.0	0	0	++++	++++

\* Sterile filtrate from cultures of  $P_{60}T_{48}$  grown in the presence of indicated amounts of penicillin.

It was observed that the filtrate from a 4-day culture of  $P_{60}T_{48}$  grown in nutrient broth plus 4 mg per ml of penicillin contained no demonstrable penicillin. This filtrate was found to contain an active penicillinase. Further experiments indicated that no penicillinase was produced in the absence of added penicillin, but an active penicillinase was produced in its presence. Table 6 presents the results of these experiments. Cultures were grown in nutrient broth for 5 days at 26 C and then filtered through glass. The culture filtrate was added in varying amounts to 10 ml of broth containing 200  $\mu$ g penicillin. Destruction of the penicillin was determined by the growth of *S. aureus* 209P.

## MORPHOLOGY

The morphological changes accompanying induced resistance to penicillin have been studied rather extensively. Since Gardner (1940) observed that exposure to penicillin was accompanied by swelling of the bacterial cells, many authors (e.g., Fisher, 1946) have confirmed and extended his observations. Klimek *et al* (1948) have shown that recently derived resistant variants of *S. aureus* 209P are larger than the parent culture and are gram-positive. When

the resistance became equal to 12,500  $\times$ , however, the cells were found to be smaller gram-negative pleomorphic cocci and bacilli. Reversal to the original gram-positive character was obtained by transferring to a penicillin-free medium. It was later found that 24 serial transfers at a penicillin concentration greater than 1 mg per ml produced a variant that retained its gram-negative character as well as its resistance to penicillin. Fifty-four serial transfers in broth without penicillin have failed to reverse the gram stain or to decrease the penicillin resistance.

A transfer of  $P_{60}T_{34}$  was sent to E F Gale to be included in his study of the relationship between amino acid assimilation and drug sensitivity (Gale and Taylor, 1946, Gale, 1947a, Gale and Taylor, 1947, Taylor, 1947). Gale has reported (1947b) that growth through two serial transfers in a deficient medium will reverse the gram stain from negative to positive. His deficient medium was

TABLE 7  
*Serial transfer for reversal of the gram stain ( $P_{60}T_{34}$ )*

MEDIUM	GRAM STAIN
Nutrient broth + 4 mg per ml penicillin	Gm-, short rods and cocci
↓	
Sufficient broth*	Gm-, short rods and cocci
↓	
Deficient broth†	Gm-, large cocci
↓	Gm+, diplococci
Deficient broth	Gm+, diplococci
↓	Gm-, large cocci (few)
Nutrient agar	Gm+, staphylococci

\* Sufficient broth, 1% yeast extract, 1% "N-Z case," 0.5%  $K_2HPO_4$ , 0.1% glucose

† Deficient broth, 0.05% yeast extract, 0.5% glucose, 0.5%  $K_2HPO_4$ , 1 mg%  $B_1$ , 1 mg% nicotinic acid

of the following composition "0.1% hydrolysed marmite, Stephenson's salt mixture, glucose, and growth factors"

We have found that the series of transfers indicated in table 7 will produce a striking reversal of the gram stain, as well as a return to the original staphylococcal forms and groupings, and a loss of most of the resistance to penicillin. Further studies are in progress to determine other properties of the reverted variants.

#### DISCUSSION

It is redundant to say that an aerobic, gram-negative rod that ferments only glucose, synthesizes nicotinic acid, and produces extracellular penicillinase would not be classified as a *Staphylococcus*. Whether such extreme changes are the result of either mutation as described by Demerec (1945) and Luria (1947), or of adaptation after the manner of Hinshelwood (1946), cannot be settled at this time. However, since adaptive functions are readily lost, the persistence

of penicillin resistance, after the removal of penicillin from the medium, could be interpreted as favoring the explanation that mutation has occurred. In a like manner the gain of ability to synthesize nicotinic acid, while growing in a medium containing sufficient nicotinic acid, and the persistence of this ability after the removal of penicillin cannot be interpreted as adaptation in the usual sense. Demerec (1945) considers that penicillin does not influence the rate of mutation but merely acts as a selective agent by preventing the growth of the more sensitive cells. The fact that cells of any desired resistance are not obtained by single transfers of a large inoculum is explained by postulating successive mutations from moderately resistant cells. One could explain the absence of nicotinic acid synthesizers in a normal culture of *S. aureus* by the same reasoning, namely, that only penicillin-resistant cells are able to mutate to nicotinic acid producers. It seems more plausible, however, to assume that penicillin influences the rate of mutation as do X-rays and certain sulfhydryl reagents (Auerbach *et al.*, 1947). At this time one can only speculate as to whether the return to the original *Staphylococcus* characteristics, upon growth in a medium deficient in amino acids and most growth factors, is the result of adaptation, spontaneous mutation, or induced mutation. Irrespective of genetic implications, however, the complete change in morphology and physiology brought about by induced resistance to penicillin, and its reversal by growth in a deficient medium, offers an excellent opportunity to study the mode of action of penicillin.

#### SUMMARY

Variants of *Staphylococcus aureus* resistant to 1 to 4 mg of penicillin per ml retain this resistance after 60 serial transfers in the presence of these concentrations of penicillin. Fifty-four subsequent serial transfers through broth in the absence of penicillin did not decrease the resistance. The variant has lost the ability to produce acid from lactose, sucrose, maltose, mannitol, and galactose. It will not grow in the presence of 6.5 per cent sodium chloride nor will it reduce nitrates. It has gained the ability to synthesize enough nicotinic acid for growth. It produces an extracellular penicillinase when grown in the presence of penicillin.

Serial transfers through a medium consisting of glucose, salts, nicotinic acid, thiamine, and 0.05 per cent yeast extract will cause a reversion from gram-negative rods to the original gram-positive staphylococcus forms. The reverted staphylococci have lost most of their resistance to penicillin.

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# AMINO ACID METABOLISM OF PENICILLIN-RESISTANT STAPHYLOCOCCI

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Studies on the assimilation of amino acids by bacteria have shown that the gram-positive cocci are able to assimilate certain amino acids and concentrate them in the free state in the internal environment prior to metabolism (Gale, 1947a, b, Taylor, 1947). The addition of penicillin to growing cultures of *Staphylococcus aureus* produces a progressive impairment of the ability of the cells to assimilate glutamic acid (Gale and Taylor, 1947), and as the culture is trained to grow in increasing concentrations of penicillin, it is found that its assimilation affinity toward glutamic acid steadily decreases (Gale, 1947c). These results suggest that, when growing in very high concentrations of penicillin, the organism adopts a different form of amino acid metabolism.

While these studies were in progress Bellamy and Klimek (1947) reported that a strain of *S. aureus* had been trained so as to increase its normal penicillin resistance by a factor of 60,000 and that the resistant variant had changed certain of its characteristics, becoming, among other things, a strict aerobe. At this time we had trained our *S. aureus* 6773 to grow in 2,000 units of penicillin per ml of medium, and at this level the culture appeared normal in colony form and staining characteristics. Following the report of Bellamy and Klimek (1947) we continued to increase the resistance of our organism and, at the level of 6,000 units of penicillin per ml, succeeded in isolating an organism similar to that described by these authors. We then entered into an arrangement with Dr. Bellamy to study the amino acid metabolism of the resistant organisms, and our findings are reported in this communication. The resistant organisms do not differ markedly from the sensitive parent strains in their ability to catabolize amino acids, but have, on the other hand, the ability to synthesize all their amino acid requirements for growth. The details of other properties of the organisms are published in the previous papers (Bellamy and Klimek, 1948a,b, Klimek *et al.*, 1948).

## ORGANISMS USED

*Staphylococcus aureus* 209 stock strain from the American Type Culture Collection, forwarded to us by Dr. Bellamy.

Organism 209 (P<sub>60</sub>T<sub>35</sub>) resistant organism derived from *S. aureus* 209 by serial subcultivation in increasing concentrations of penicillin, isolated by Bellamy and Klimek (1947).

*Staphylococcus aureus* 6773 strain isolated from nasal swab by Dr. B. Topley and used by us for assimilation studies (Gale, 1947b).

*Staphylococcus aureus* 6773 (2000) strain derived from *S. aureus* 6773 by serial subcultivation in increasing concentrations of penicillin per ml of medium

Organism 6773(PT) organism derived from *S. aureus* 6773 (2000) by subcultivation first in 4,000 units of penicillin per ml and then in 6,000 units per ml

The strains of *S. aureus* 209, 6773, and 6773 (2000) are normal organisms of this species and are resistant to 0.08, 15, and 2,000 units of penicillin per ml, respectively. Organisms 209(P<sub>60</sub>T<sub>35</sub>) and 6773(PT) are gram-negative, pleomorphic, and strict aerobes (Bellamy and Klimek, 1948a, b)

#### BREAKDOWN OF AMINO ACIDS

**Methods** The organisms were grown on the surface of casein digest agar for 20 hours at 37 C, washed off the agar with distilled water, centrifuged down, washed once, and made up into suspension in distilled water. The dry weight of the cell suspensions was determined turbidimetrically in a Hilger absorptiometer previously calibrated against known suspensions of the organisms concerned.

The oxidation of amino acids was followed in Warburg manometers shaken in a thermostat at 37 C, the cups contained 1.0 ml washed suspension and 1.5 ml M/5 phosphate buffer pH 7.0 in the main cup, 0.5 ml M/10 amino acid solution at pH 7.0 in the side bulb, and 0.2 ml 10 per cent NaOH in the center cup. Rates of oxidation were studied over a period of 1 hour after the addition of the substrate, and activities are expressed as  $Q_{O_2}$  =  $\mu$ l O<sub>2</sub> uptake per hr per mg dry weight of cells.

Ammonia formation was determined on the contents of the manometer cups at known intervals. Two ml of the cup contents were removed and made alkaline with saturated K<sub>2</sub>CO<sub>3</sub>, and the ammonia was distilled into acid in Conway vessels. The ammonia was estimated by the addition of Nessler's reagent followed by estimation in a photoelectric colorimeter. Results are expressed as  $Q_{NH_3}$  =  $\mu$ l NH<sub>3</sub> liberated per hr per mg dry weight of cells. Values for oxidation and ammonia production are corrected for blanks without amino acid.

**Experimental results** The results obtained are summarized in table 1. In general there are no marked differences between the activities of the organisms from the parent penicillin-sensitive cultures and of those from the resistant cultures. In some cases, e.g., glycine, serine, proline, and glutamic acid, the rates of attack by the resistant organisms are significantly lower than by the parent strains, and this can perhaps be correlated with the finding that the resistant organisms grow more slowly than the parent strains (Bellamy and Klimek, 1947). In other cases, e.g., arginine and lysine, the resistant organisms have a significantly higher rate of attack than do the parent strains. The rates are low in all cases, with the exception of glycine, and the values are in general agreement with those found by Hills (1942) for a variety of *Staphylococcus*. The only case in which the rate of deamination is significantly higher than the rate of oxidation is that of arginine, for all four organisms an anaerobic

deamination with liberation of  $\text{CO}_2$  can be demonstrated in addition to the oxidative breakdown, and it is probable that the greater ammonia formation is due to the action of arginine dehydrolase (Hills, 1942)

There was no anaerobic deamination of serine or aspartic acid with organism 209 ( $\text{P}_{60}\text{T}_{35}$ ), and no amino acid decarboxylases could be found when washed suspensions were tested under the normal conditions for such enzymes (Gale, 1946)

TABLE 1  
*Imino acid oxidation by staphylococci*

ORGANISM	6773		6773 (PT)		209		209 ( $\text{P}_{60}\text{T}_{35}$ )	
	$\text{QO}_2$	$\text{QN}_2$	$\text{QO}_2$	$\text{QN}_2$	$\text{QO}_2$	$\text{QN}_2$	$\text{QO}_2$	$\text{QN}_2$
Glycine	23.5	26.5	4	5	41	30	8	7
L-Alanine	9	12.5	11	6	14	9.6	16	8
L-Glutamate	5	5	1	0	9	8	1	0
Glutamine	9	9	2	1	6	6	3	4
L-Aspartate	3	6	5	0	—	—	6	2
L-Arginine	1	7	7	12	1	7	6	16
Serine	6	19	9	9	27	20	9	7
L-Lysine	1	3	8	4	1	0	13	7
L-Proline	8.5	6	6	3	14	—	4	2
Valine								
L-Leucine								
L-Phenylalanine							0	0
L-Tyrosine								
L-Histidine								
L-Tryptophane								
(Blank)	22	—	17	—	18	—	7	—
(Glucose)	19	—	8	—	54	—	34	—

Organisms grown on the surface of casein digest agar for 20 hours at 37 C

Activities expressed as  $\text{QO}_2 = \mu\text{l O}_2$  uptake per hr per mg dry weight of cells at pH 7.0 and 37 C,  $\text{QN}_2 = \mu\text{l NH}_3$  liberated per hr per mg dry weight of cells at pH 7.0 and 37 C

#### ASSIMILATION OF GLUTAMIC ACID

**Methods** The assimilation of glutamic acid by suspensions of the cells grown in "deficient" media was studied by the methods previously described (Gale, 1947a, c)

**Experimental results** Table 2 shows the final equilibrium concentration of free glutamic acid attained within 100 mg dry weight of cells when these have been suspended in a solution of glutamic acid (200  $\mu\text{l}$  per ml), glucose (1 per cent), and crystal violet (1  $\mu\text{g}$  per mg cells). The addition of crystal violet inhibits internal metabolism of the amino acid (Gale and Mitchell, 1947) so that the level of free glutamic acid measured within the cell is a measure of assimilation without complication owing to metabolism. The gram-positive cells show the usual high concentration of free glutamic acid within the internal environment (Taylor, 1947), but no free glutamic acid can be demonstrated in the internal

environment of either of the resistant gram-negative organisms. These results thus conform to the finding of Taylor (1947) that only gram-positive bacteria are able to effect an internal concentration of free amino acids. It has been shown previously (Gale, 1947c) that the assimilation affinity (= reciprocal of assimilation constant) of the cells toward glutamic acid decreases as their resistance to penicillin increases. The assimilation constants of *S. aureus* 209, 6773, and 6773(2000) are 2, 12, and 50, respectively, expressed in  $\mu$ l of glutamic acid per ml of external medium (Gale, 1947c). Since no free internal glutamic acid can be measured in 209 ( $P_{60}T_{35}$ ) and 6773(PT), it is not possible to determine their assimilation affinities.

TABLE 2  
Assimilation and internal concentration of free glutamic acid

ORGANISM	INTERNAL CONCENTRATION $\mu$ l GLUTAMIC ACID PER 100 MG OF CELLS
<i>S. aureus</i> 209	561
Organism 209 ( $P_{60}T_{35}$ )	0
<i>S. aureus</i> 6773	825
<i>S. aureus</i> 6773 (2000)	705
Organism 6773 (PT)	0

Cells are grown in salt mixture + 0.1% marmite + 1% glucose for 6 hours at 37°C, made up into washed suspension, and incubated for 1 hour at strength 1 mg per ml in buffered salt solution + glutamic acid (200  $\mu$ l per ml) + glucose (1%) + crystal violet (1  $\mu$ g per ml), and the internal level of free glutamic acid is determined (Gale, 1947b).

#### SYNTHESIS OF AMINO ACIDS

**Methods** The growth requirements of the organisms were studied using the salt mixture, growth factors, and amino acids shown to be necessary for *Staphylococcus aureus* by Gladstone (1937), all nutrients were used in the concentrations recommended and sterilized by the methods adopted by Gladstone. To prepare the inoculum of each organism, the growth from 24-hour cultures on casein digest agar was washed off with sterile distilled water and the suspension standardized to approximately  $2 \times 10^5$  organisms per ml by comparison with opacity tubes, one drop of this suspension was then used as standard inoculum for 10 ml of medium. When satisfactory growth was obtained in a complete synthetic medium, the culture was then inoculated into this medium, from which each amino acid and growth factor was omitted in turn. In this way, those amino acids and growth factors essential for growth in the otherwise complete medium were determined. In those cases in which growth did not take place the tubes were left for a period up to 163 hours at 37°C in order to determine whether adaptation by training would occur.

**Experimental results** Table 3 shows the effect of omitting amino acids and growth factors one at a time from the complete medium for each of the five organisms. With the exception of *S. aureus* 209, which requires 40 hours, all

organisms grew within 24 hours in the complete medium containing all the amino acids, nicotinamide, and thiamine. Omission of the amino acids one at a time had no effect on the growth of the two resistant organisms, and they were subsequently found to give maximal growth after some lag in a medium consisting of salts, ammonium sulfate, glucose, cystine, and thiamine, and to give delayed and submaximal growth in the same medium without cystine. The power of the organism 209(P<sub>60</sub>T<sub>35</sub>) to synthesize nicotinamide has been noted also by Bellamy

TABLE 3  
*Nutritional requirements of staphylococci*

ORGANISM	6773	6773 (2000)	6773 (PT)	209	209 (P <sub>60</sub> T <sub>35</sub> )
Nicotinamide	+	+	—	+	—
Thiamine	+	+	+	+	+
Proline	(88)	(88)	—	+	—
Histidine	+	+	—	+	—
Valine	+	+	—	+	—
Glycine	+	(88)	—	+	—
Aspartate	+	(64)	—	+	—
Leucine	(40)	(40)	—	+	—
Cystine	+	+	—	+	—
Glutamate	(40)	—	—	—	—
Phenylalanine	(40)	(64)	—	—	—
Arginine	+	+	—	—	—
1 Growth in complete medium in	24 hr	24 hr	24 hr	40 hr	24 hr
2 Growth in salts, ammonium sulfate, glucose, growth factors, and cystine	nil	nil	64 hr	nil	64 hr
3 Growth in medium 2 less cystine	nil	nil	88 hr	nil	88 hr

+ = presence essential for growth in same time as in complete medium

— = presence not essential for growth in same time as in complete medium

(88) = in absence of amino acid growth equal to that in complete medium occurs in 88 hours

All media contain salt mixture, ammonium sulfate, and glucose as in Gladstone (1937). Incubation is at 37 C. All amino acids are added as the natural isomers with the exception of valine, which was used in the racemic form.

and Klimek (1948a,b). Both parent strains require a range of amino acids for growth, with strain 6773 the omission of any of 10 amino acids, and with strain 209 the omission of any of 7 amino acids, from the complete medium resulted in delayed or complete absence of growth within the experimental period. Strain 6773 is able to adapt to the synthesis of glutamic acid, phenylalanine, and leucine in 40 hours and to proline in 88 hours. The strain 6773(2000) occupies an interesting position intermediate between that of 6773 and that of the resistant non-exacting 6773(PT). It is able to adapt to the omission of glycine in 88 hours and

of aspartic acid in 64 hours, whereas the parent strain 6773 is unable to grow in the absence of either of these amino acids within the longest experimental period used of 163 hours, also, 6773(PT) is nonreacting toward glutamic acid, but the parent strain will not grow in the absence of this amino acid until adaptation has occurred after a lapse of 40 hours

#### DISCUSSION

The ability to concentrate amino acids in the free state in the internal environment is a property specific to gram-positive bacteria (Taylor, 1947), and many of these organisms have lost the power to synthesize certain of their amino acid requirements. It may be that this power of internal concentration is a mechanism evolved in compensation for the loss of synthetic ability. It has previously been shown that the action of penicillin is to impair the assimilatory process toward glutamic acid (Gale and Taylor, 1947) and that training to increased penicillin resistance is accompanied by decreasing ability to assimilate glutamic acid (Gale, 1947c). At the higher levels of penicillin resistance the assimilation affinity toward glutamic acid decreases very rapidly, and it is at these high levels that we find the marked alteration in properties of the cultures described here and in the previous papers.

Demerec (1945) has shown that penicillin training consists of the selection of resistant mutants within the culture. Penicillin impairs the ability of gram-positive cells to assimilate certain free amino acids, and it would appear that the training process results in the selection of mutants that are less dependent on the assimilation process until, eventually, all organisms are suppressed except those that grow by synthesis instead of by assimilation of amino acids. The resistant organisms described here have the power to synthesize all their amino acid requirements, which suggests that the effect of penicillin in suppressing the assimilation of glutamic acid is symptomatic of changes in the general assimilatory properties of the cells. The fact that these cells, which can synthesize but not concentrate amino acids, are gram-negative again suggests that the ability to concentrate free amino acids in the internal environment is associated with the presence of the gram complex in the cell wall.

#### SUMMARY

The amino acid metabolism of two organisms rendered resistant to high levels of penicillin has been compared with that of the penicillin-sensitive parent strains (*Staphylococcus aureus*). The resistant organisms do not differ markedly from the parent strains in their ability to oxidize amino acids, but they do differ in that they have no power to concentrate free glutamic acid in the internal environment and are able to synthesize all their amino acid requirements from ammonia and glucose in the presence of thiamine.

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## THE NUTRITION OF PROTOZOA

### III AN IMPROVED PROCEDURE FOR SEPARATING HUMAN BLOOD SERUM INTO THE TWO FRACTIONS ESSENTIAL FOR THE SUSTAINED GROWTH OF *TRICHOMONAS VAGINALIS*

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Very little is as yet known about the factors in blood serum necessary for the sustained growth of parasitic protozoa. Preceding publications from this laboratory (Sprince and Kupferberg, 1947a,b) have shown that the parasitic protozoan, *Trichomonas vaginalis*, could be conveniently used in a suitable basal medium as an assay organism for such factors. A procedure for the ether separation of human serum into an ether-soluble fraction and an ether-insoluble residue both essential for the sustained growth of the organism was presented.

Although successful fractionations could be accomplished, the method was not without some inherent shortcomings (Sprince and Kupferberg, 1947b). In attempting to inaugurate a program of routine fractionations, frequently the degree of completeness of the separation was questionable as manifest by residual growth in the ether-insoluble phase per se through several serial transfers, in other cases, fractionation attempts resulted in third layer formation (protein precipitation) and subsequent extracts that could not be made to pass through a bacteria-retaining sterile filter.

The earlier observations of Hardy and Gardiner (1910) and McFarlane (1942) suggested to us that minute differences in the alcohol content of the ether employed had a marked effect on the completeness of separation and filterability of the fractions obtained. Extraction experiments with various grades of ether verified this. Thus, use of ethyl ether U.S.P. no. 10 (Merck) containing 3.5 per cent alcohol resulted in fractions with third layer formation (protein precipitation). Absolute ethyl ether (Merck) containing 0.1 per cent or less of alcohol<sup>1</sup> yielded reasonably filterable active fractions, although frequently the separation was not complete. In view of these peculiar difficulties and the repeated observations that alcohol-ether mixtures must be utilized for the complete extraction of lipids from serum (Hardy and Gardiner, 1910, McFarlane, 1942, Hewitt, 1927, Horsfall and Goodner, 1935, Cappo, 1941), it was felt that an improved method of fractionation was in order.

In the work now presented such a method is described in detail, and further steps in the isolation of the active materials are outlined.

<sup>1</sup> This grade of alcohol was used in work described in our previous publications (Sprince and Kupferberg, 1947a,b).

## EXPERIMENTAL PROCEDURES

The principle upon which this procedure was based was one of controlling the proportions of alcohol and ether used during the fractionation process so that a maximum extraction of serum lipids could be effected without precipitating the residual serum proteins. All extractions and precipitations were made at room temperature.

*Alcohol-ether separation* Two hundred ml of human blood serum (pooled from negative Wassermann tests) were adjusted to pH 7.1 to 7.3 with 1 N HCl or 1 N NaOH by means of the glass electrode. Thirty ml<sup>2</sup> of absolute alcohol (Commercial Solvents, 200 per cent proof) were added drop by drop with constant stirring to avoid protein precipitation. A clear solution resulted if the mixture was stirred well. Eight to 12 extractions with 400-ml portions of absolute ethyl ether (Mallinckrodt, containing 0.01 per cent alcohol or less) were made in a separatory funnel as described in a previous publication (Sprince and Kupferberg, 1947b).

*Ether extract* The ether extracts were pooled, evaporated to dryness, and made up to a 200 ml ( $\approx$  200 ml intact serum) aqueous emulsion as described previously (Sprince and Kupferberg, 1947b). To facilitate sterile filtration, 0.2 ml to 0.4 ml of "tween 80" were added. The addition of "tween 80" in these concentrations has been shown to have no appreciable effect on the growth or inhibition of our strain of *Trichomonas vaginalis*. The mixture was shaken well until the "tween 80" was completely dissolved, and then it was rendered sterile by being passed through a bacteria-retaining sterile filter. This fraction was then stored in 50-ml lots in the cold.

*Ether-insoluble phase* The alcohol and ether were evaporated off from the ether-insoluble phase by the procedure used by Sprince and Kupferberg (1947b). The pH was checked and found to be the desired value, 7.2. The material was now made up to 200 ml in volume with distilled water ( $\approx$  200 ml intact serum) and divided into two 100-ml portions. One 100-ml portion was sterilized by filtration and stored in 50-ml lots in the cold. This aliquot was assayed for activity and represented the complete ether-insoluble phase. The second 100 ml portion was subjected to further fractionation as follows.

*Alcohol precipitate of the ether-insoluble phase* To 100 ml of the ether-insoluble phase prepared above, 100 ml of absolute alcohol (Commercial Solvents, 200 per cent proof) were added slowly with stirring, thereby forming a 50 per cent alcohol solution<sup>3</sup>. A flocculent proteinaceous precipitate resulted. The precipitate was centrifuged off, washed first with three 300-ml portions of 50 per cent alcohol (prepared from absolute alcohol), and then washed with three 300-ml portions of distilled water. The centrifugate and all washings were

<sup>2</sup> This volume of alcohol (13 per cent with respect to serum) was the maximum amount of alcohol that could be added to serum without protein precipitation.

<sup>3</sup> Fifty per cent alcohol was the minimum alcohol concentration that completely precipitated all proteinaceous material from serum. No further precipitation resulted on the addition of more alcohol.

pooled and kept in the cold for further treatment. The washed precipitate was now suspended in 70 ml of distilled water, and 5 N NaOH was added drop by drop with constant vigorous shaking until a clear, freely flowing solution resulted. The pH at this point may vary from 10.5 to 11.3. In order to prevent excessive protein denaturation at this high pH, *immediately* after such a solution was attained, the pH was readjusted drop by drop with 1 N HCl to a range of 7.0 to 7.3. The solution remained clear and filterable. The material was now adjusted to 100-ml volume ( $\approx$  100 ml intact serum) with distilled water, sterilized by filtration, and stored in the cold in 50-ml lots prior to assay. This fraction contained all proteins precipitable by 50 per cent alcohol.

*Alcohol filtrate of the ether-insoluble phase.* The pooled centrifugate and washings obtained above were evaporated *in vacuo* to approximately 80 to 90 ml. The pH was brought to 7.2 with 1 N NaOH. The solution was adjusted to 100-ml volume ( $\approx$  100 ml intact serum) with distilled water, sterilized by filtration, and stored in the cold in 50-ml lots until ready for assay. This fraction contained all the water-soluble compounds of serum not precipitable by 50 per cent alcohol.

*Assay.* Assays of all fractions were performed in trypticase basal medium by means of a washed inoculum from a pure culture of the protozoan *Trichomonas vaginalis*, strain no. 2,<sup>4</sup> cultured in trypticase-serum medium. The treatment of the inoculum, the preparation of serum fractions for assay, and the basal medium used in such assays have been presented in preceding publications (Sprince and Kupferberg, 1947a, b).

## RESULTS

The results of assays of fractions prepared by the foregoing procedures are depicted in table 1.

It is evident that no one fraction alone could maintain sustained growth to any marked extent. No growth was demonstrable in either the ether-insoluble phase, the alcohol precipitate of the ether-insoluble phase, or the alcohol filtrate of the ether-insoluble phase when these were assayed by themselves. Only one fraction, viz., the ether extract, showed any appreciable cell count. This, however, was probably due to the residual growth of organisms originating from a vigorously growing inoculum when placed in a medium capable of maintaining survival. It is interesting to note that, of all the fractions tested, the ether extract alone (prepared by this new procedure) was capable of maintaining survival through three serial transfers from a washed inoculum. This effect could not be attributed to small amounts of residual protein carried into the ether extract during the separation. The aqueous emulsion of the ether extract used in the assay was shown to be free of protein by its giving a negative result to each of the following tests: heat (autoclaving at 15 pounds for 15 minutes), alcohol precipitation (absolute alcohol), phosphotungstic acid, trichloroacetic acid, xanthoproteic, picric acid, biuret, Erlich's benzaldehyde reagent, Folin's phenol

<sup>4</sup> The use of cultures of *Trichomonas vaginalis*, strain no. 2, obtained from Dr. Garth Johnson and Mr. A. B. Kupferberg of the Department of Microbiology, Ortho Research Foundation, is gratefully acknowledged.

reagent after alkaline hydrolysis, and the triketohydrindene reagent after alkaline hydrolysis and subsequent neutralization

The absence of organisms in the unfractionated ether-insoluble phase was especially significant. This effect was apparent upon microscopic examination immediately after the first culture from the original washed inoculum, and served to indicate that the removal of the *active* serum lipids from the residual protein was well accomplished. The completeness of the separation was further

TABLE 1

*Growth-promoting activity for Trichomonas vaginalis of fractions prepared from human serum*

(Each value is the average of duplicate determinations. The data for two different extractions are presented)

MATERIAL ADDED	GROWTH OF TRICHOMONAS VAGINALIS EXPRESSED IN NUMBER OF CELLS PER MM <sup>2</sup> AT END OF 2 SERIAL TRANSFERS	
	Extraction 1	Extraction 2
None (basal trypticase control)	0	0
Human intact serum (0.5 ml) (control)	1,365	1,305
EE* (0.5 ml)	92	75
ER† (0.5 ml)	0	0
ERP‡ (0.5 ml)	0	0
ERF§ (0.5 ml)	0	0
EE (0.5 ml) + ER (0.5 ml)	1,500	2,100
EE (0.5 ml) + ERP (0.5 ml) + ERF (0.5 ml)	1,540	1,055
EE (0.5 ml) + ERF (0.5 ml)	0	0
EE (0.5 ml) + ERP (0.5 ml)	1,400	1,475

All samples of intact serum or serum fractions were diluted with an equal volume of Ringer's solution (Sprince and Kupferberg, 1947b). Sterile 0.5 ml samples of such materials were pipetted into 8 ml of sterile trypticase basal medium, and enough sterile Ringer's solution was added to bring the final volume in each tube to 10 ml (Sprince and Kupferberg, 1947a). Inocula were cultured in trypticase serum medium and washed twice with sterile saline solution before assay. For the sake of convenience, the first culture was allowed to proceed for 72 hours. Two serial transfers were then made at 48 hour intervals. Cell counts were made by hemocytometer.

\* Ether extract fraction

† Ether-insoluble phase (ether residue)

‡ Alcohol precipitate of the ether-insoluble phase

§ Alcohol filtrate of the ether-insoluble phase

emphasized by a comparison of the dry weights (dried to constant weight at 80 C *in vacuo*) of the ether extract prepared by the method described above, as contrasted with the procedure in our previous publication (Sprince and Kupferberg, 1947b) wherein Merck's absolute ether (containing 0.1 per cent alcohol) was used. By our present procedure on a given lot of serum, 732 mg of lipoidal material per 100 ml of serum could be extracted as compared with 312 mg per 100 ml by the earlier method.

From table 1, it will be observed that when both the aqueous emulsion of the ether extract and the aqueous solution of the solubilized alcohol precipitate of

the ether-insoluble phase were added simultaneously, growth occurred to a degree *equivalent* to intact serum. This indicated that all of the active components of the ether-insoluble phase were contained in the protein fraction. Inasmuch as during the process of separation, the proteins probably had undergone some degree of denaturation (Edsall, 1947) it would be of interest to learn how far, by other forms of treatment, denaturation or degradation of the protein fraction could be permitted to proceed and still allow for a retention of its activity. Future experiments are contemplated along this line. In this connection it should be noted, parenthetically, that sustained growth with restricted cell counts (approximately 560 cells per mm<sup>3</sup>) could be obtained by substituting *intact* serum albumin for the ether-insoluble phase (Sprince and Kupferberg, 1947b).

#### DISCUSSION

The procedure for the fractionation of human serum outlined in this paper was developed primarily with respect to the separation of factors essential for the sustained growth of *Trichomonas vaginalis*. Two fractions were obtained (1) a lipid fraction that was shown to be free from protein, and (2) a protein fraction that contained all the proteins in serum precipitable by 50 per cent alcohol. Both of these fractions were necessary in order to obtain growth of the organism in numbers equivalent to that in intact serum. The purpose that motivated the development of this procedure is not to be confused with that which motivated fractionation procedures recently developed by Cohn and his associates at Harvard (1946). These investigators have been chiefly concerned with resolving the plasma and serum proteins into their purified protein components.

Attempts to obtain growth with certain serum fractions prepared by the Plasma Fractionation Laboratory at Harvard<sup>5</sup> were unsatisfactory since not all fractions were available to allow for the compounding of a completely reconstituted serum. Similarly, lyophilized intact bovine serum<sup>6</sup> as well as a "synthetic" bovine serum, reconstituted from lyophilized bovine serum fractions,<sup>6</sup> I, II + III, IV, V, and VI, gave irregular results. In all cases, various degrees of difficulty were encountered by loss of material on the filter during sterile filtration. In this connection, see Cohn *et al* (1946) note 72. Further experiments, however, are contemplated along these lines.

The method described above has several advantages over other fractionation procedures reported in the literature. Relatively large quantities of serum may be fractionated at room temperatures with simple laboratory apparatus. The fractions obtained pass readily through bacteria-retaining sterile filters and possess a high degree of activity. From the standpoint of residual growth-promoting ability for *Trichomonas vaginalis*, separation is excellent.

<sup>5</sup> We are indebted to Dr. John Edsall of the Department of Physical Chemistry, Harvard Medical School, for furnishing us with samples of various fractions prepared from human serum.

<sup>6</sup> We wish to thank Dr. J. D. Porsche, Research Division, Armour Laboratories, for generous amounts of lyophilized intact bovine serum and serum fractions.

## SUMMARY

An improved procedure for separating human blood serum into two fractions both essential for the sustained growth of *Trichomonas vaginalis* is outlined. The principle upon which this method was based was the control of the proportions of alcohol and ether used during the fractionation process so that a maximum extraction of serum lipids could be effected without precipitating the residual serum proteins. Two fractions were obtained (1) a lipid fraction that was shown to be free from protein, and (2) a protein fraction that contained all the proteins in serum precipitable by 50 per cent alcohol. Both of these fractions were necessary in order to obtain growth of the organism in numbers equivalent to that obtained with intact serum.

Several advantages are evident in the procedure outlined. Relatively large quantities of serum may be fractionated at room temperatures with simple laboratory apparatus. The fractions obtained pass readily through bacteria-retaining sterile filters and possess a high degree of activity. Separation of the fractions from the standpoint of the *Trichomonas vaginalis* assay is excellent.

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## THE REVERSAL OF ANTIBIOTIC ACTION

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Previous reports from this laboratory (Cavallito and Bailey, 1944, Cavallito *et al.*, 1945) have shown that many antibiotics may be inactivated with various thiol compounds, particularly cysteine. We have interpreted this as indicating that a large group of antibacterial agents acts by combining with protein sulfhydryl groups essential to growth. Barron and Singer (1945) and Singer and Barron (1945) have shown that many of the enzymes involved in growth and respiration contain this group or require it for their activation. Hellerman *et al.* (1943) have shown the importance of  $-SH$  in the activity of urease. These workers have shown that crystalline urease may be inactivated by mercuric chloride and the activity restored by treatment of the inactivated enzyme with cysteine.

Data are here presented that show that treatment of certain bacteriostatic systems with thiol compounds, particularly cysteine, will reverse bacteriostasis, i.e., will allow the inhibited bacteria to take up oxygen again and multiply in the presence of a bacteriostatic concentration of the antibacterial agent.

Eight antibiotics have been studied for the property of reversibility: penicillin, streptomycin, pyocyanin, the active principle of *Asarum canadense* (Cavallito and Bailey, 1946), phenyl mercuric acetate, mercuric chloride, gliotoxin, and the active principle of *Allium sativum* (allyl 2-propene-1-thiosulfinate, referred to hereafter as the thiolsulfinate).

The technique generally employed to show reversal of bacteriostasis was as follows: the culture was prepared by adding 0.1 ml of an 18- to 20-hour culture in beef extract broth to 50 ml of sterile beef extract broth and incubating, depending on the organism employed, for 2 or 3 hours at 37°C. Two-ml portions of the 2-hour culture were placed in the reaction chamber of sterile Warburg flasks having 40 per cent KOH in the center well. The antibacterial agent was added in 0.5-ml portions to the reaction chamber of all the vessels excepting those to be used for culture control, which received a similar volume of phosphate buffer of pH 6.5. The thiol compounds were placed in the side arms of the Warburg flasks in the experimental vessels, whereas the control vessels contained phosphate buffer of pH 6.5 or sterile distilled water. Oxygen uptake in air by the bacteria was determined by the usual Warburg technique, observations being made every 10 minutes.

The antibacterial agents were prepared in the following concentrations: thiolsulfinate, 0.06 mg per ml, mercuric chloride, 0.01 mg per ml, active principle of *Asarum canadense*, 0.06 mg per ml, penicillin, 0.036  $\mu$ g per ml, pyocyanin, 0.12 mg per ml, gliotoxin, 0.01 mg per ml, streptomycin, 24  $\mu$ g per ml, and phenyl mercuric acetate, 0.0012 mg per ml. These concentrations are below the bactericidal levels of the agents for the organisms used.

Of the antibiotics tested, the bacteriostatic action of mercuric chloride, gliotoxin, and the thiolsulfinate could be reversed. The results of an experiment using the thiolsulfinate and *Salmonella paratyphi* that demonstrates this characteristically for the group may be described in greater detail. The results are summarized in table 1. In this experiment the vessels of the Warburg were divided into 5 groups (A, B, C, D, and E) of 3 vessels each. The reaction chambers contained the culture and antibacterial agent as described above, the vessels

TABLE 1

*Reversal by cysteine of the bacteriostatic action of allyl 2-propene-1-thiolsulfinate on Salmonella paratyphi A*

Interval	CONTENTS OF VESSELS  <i>ml</i>	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20-MINUTE INTERVAL				
		VESSEL GROUP				
		A	B	C	D	E
	Culture	2 0	2 0	2 0	2 0	2 0
	Antibiotic	0	0 5	0 5	0 5	0 5
	Water	0 5	0 5	0	0	0
	Side arm Cysteine	0 5	0	0 5	0 5	0 5
Control vessels tipped		21 9	0	7 1	0 4	3 3
1st Per		70 0	8 0	28 1	10 7	21 3
2nd Per		88 7	1 2	21 4	11 9	21 3
3rd Per		97 7	6	18 8	17 1	31 3
4th Per		135 4	0	23 8	31 6	49 4
5th Per		89 9	0	36 0	56 9	43 4
6th Per		83 2	6	41 0	64 4	52 4
7th Per		91 4	1 8	54 8	92 6	63 9
8th Per		99 1	3 1	84 1	95 9	88 1

Values are averages of 3 vessels

Culture a 2-hr culture of *Salmonella paratyphi* A in beef extract broth

Antibiotic a 0.06 mg per ml solution of the thiolsulfinate in distilled water

Cysteine solution in groups A and C, 5.6 mg per ml, in group D, 0.56 mg per ml, and group E, 0.28 mg per ml

of group A (culture control) having sterile distilled water in place of the antibacterial agent. The side arms of groups A, C, D, and E contained 0.5 ml of cysteine solutions of the following concentrations: A and C, 5.6 mg per ml, D, 0.56 mg per ml, E, 0.26 mg per ml. The side arms of group B (bacteriostasis control) contained a similar volume of sterile distilled water. The cysteine hydrochloride solutions were prepared and sterilized by filtration through sintered glass filters just prior to use, and were then neutralized with solid sodium bicarbonate. After observations had been made to show oxygen uptake in the culture controls and lack of consumption in the bacteriostasis control and the experimental groups, the cysteine was tipped into the vessels and oxygen uptake again measured. To conserve space the results are expressed for 20-minute ob-



servation periods, although the determinations were made every 10 minutes. It is at once obvious that the control culture was respiring and that the concentration of the thiolsulfinate present in the test vessels had inhibited oxygen uptake. Upon the addition of the cysteine there was prompt oxygen uptake by the cultures to which it had been added. That this is not due to mere dilution is shown by the fact that the bacteriostasis control was diluted to the same extent, with no resumption of respiration. At the end of the experiment all vessels except group B (bacteriostasis control) were quite turbid. The speed with which oxygen uptake occurred shows that the resumption of respiration is

TABLE 2

*Lack of reversal of the bacteriostatic action of the active principle of Asarum canadense on Staphylococcus aureus*

Interval	CONTENTS OF VESSELS  ml	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20-MINUTE INTERVAL			
		VESSEL GROUP			
		A	B	C	D
	Culture	2 0	2 0	2 0	2 0
	Antibiotic	0	0 5	0 5	0 5
	Buffer	1 0	0 5	0	0
	Side arm Cysteine	0	0 5	0 5	0 5
Control vessels tipped		14 0	4 9	7 0	8 3
1st Per		50 0	8 7	9 1	8 6
2nd Per		57 8	7 4	7 0	6 3
3rd Per		81 9	5 7	5 7	5 4
4th Per		83 7	5 2	5 3	3 8
5th Per		85 8	5 2	5 3	3 3

Values are averages of 4 vessels in groups A and B and 3 vessels in C and D

Culture 3½-hour culture *Staphylococcus aureus* 209

Antibiotic a 0.06 mg per ml solution of *Asarum canadense*

Buffer a M/2 phosphate buffer pH 6.6

Cysteine solution in group C vessels, 0.94 mg per ml in buffer similar to the above, that in group D, 0.094 mg per ml in similar buffer

due to the reversal of bacteriostasis and not to the respiration of a few resistant organisms.

Data typical of those antibiotics the bacteriostatic action of which could not be reversed are presented in table 2, in which results using the active principle of *Asarum canadense* and *Staphylococcus aureus* are recorded. (Similar data were obtained with penicillin, streptomycin, and pyocyanin with *S. aureus*, and with phenyl mercuric acetate and *S. paratyphi*.) With the *A. canadense* antibiotic, no resumption of oxygen uptake was observed. The culture medium in the bacteriostasis control group vessels and that in the experimental groups showed no difference in turbidity at the end of the experiment, whereas the medium of the culture control was very turbid. This antibiotic resembles penicillin in

several respects. The *A. canadense* antibiotic is active in low concentrations against gram-positive organisms, contains sulfur, and is inactivated by alkali and by cysteine (Cavallito and Bailey, 1944). The selective action shown by penicillin on "young" cells, however, is not exhibited by this antibiotic. This is well illustrated in table 3. The test organism in this experiment was *Bacillus subtilis*. The "young" cells were a 4-hour culture prepared by inoculating 50 ml of sterile beef extract broth with 0.1 ml of a 20-hour broth culture of the test organism, whereas the "old" cells were a similar culture incubated 24 hours. The antibiotic, dissolved in 10 per cent ethanol, was placed in the side arms of the experimental vessels, in the controls an equal volume (0.5 ml) of 10 per cent ethanol

TABLE 3

*Effect of age of culture on bacteriostasis of Bacillus subtilis by the active principle of Asarum canadense*

	CONTENTS OF VESSELS	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20 MINUTE INTERVAL			
		VESSEL GROUP			
		A	B	C	D
Interval	ml				
	Culture, 24 hr	2.5	0	2.5	0
	Culture, 4 hr	0	2.5	0	2.5
	Ethanol	0.5	0.5	0	0
	Side arm Antibiotic	0	0	0.5	0.5
Control vessels tipped		28.7	18.2	22.2	19.2
1st Per		50.3	59.5	64.2	37.4
2nd Per		54.4	105.9	91.9	52.4
3rd Per		71.6	126.4	64.5	31.6
4th Per		60.9	75.7	41.2	23.6
5th Per		94.9	134.9	19.4	11.5

Values are averages, groups A and B, 3 vessels each, groups C and D, 4 vessels

Ethanol 10% solution    Concentration of active principle of *Asarum canadense*, 0.15 mg per ml in 10% ethanol

was used. When oxygen uptake was shown in all vessels, the contents of the side arms were tipped into the reaction chambers and oxygen uptake was again determined. The active principle of *Asarum canadense* is equally effective in stopping the oxygen uptake of "old" and "young" actively growing cells. At the end of the experiment the control vessels were very turbid, whereas the experimental vessels were no more turbid than at the beginning of the experiment.

The antibiotics that produced a bacteriostasis that could be reversed with cysteine (thiolsulfinate, mercuric chloride, and gliotoxin) were tested for reversal with the following thiol compounds: glycylcysteine, N-acetylcysteine, and sodium thioglycolate. Bacteriostasis produced by mercuric chloride was reversed by all of these compounds, bacteriostasis by the thiolsulfinate by all but sodium thioglycolate. Unexpectedly, only N-acetylcysteine was effective with gliotoxin. Presentation of all the data on these compounds with the three anti-

biotics would be repetitious, as examples of the effectiveness and lack of it, the data obtained with the thiolsulfinate are presented in table 4. The rate of reversal is more rapid with cysteine than with the other thiols tested. S-methylcysteine, propanethiol, and  $\beta$ -(dimethylamino)-ethanethiol were without effect in reversing the bacteriostasis of *S. paratyphi* by mercuric chloride.

TABLE 4

Action of glycylcysteine, sodium thioglycolate, and N-acetylcysteine on the bacteriostatic action of thiolsulfinate on *Salmonella paratyphi* A

Interval	CONTENTS OF VESSELS  ml	CUBIC MILLIMETERS OF OXYGEN UPTAKE PER 20 MINUTE INTERVAL				
		VESSEL GROUP				
		A	B	C	D	E
	Culture	2 0	2 0	2 0	2 0	2 0
	Buffer	0 5	0 5	0 5	0 5	0 5
	Side arm Buffer	0 5	0 5	0	0	0
	Glycylcysteine	0	0	0 5	0	0
	Na-thioglycolate	0	0	0	0 5	0
	N-acetylcysteine	0	0	0	0	0 5
Control vessels tipped		3 1	1 2	1 7	2 9	1 9
1st Per		12 1	1 5	4 5	1 0	2 0
2nd Per		18 8	1 8	1 7	2 9	6 0
3rd Per		35 5	2 2	5 4	0 5	6 6
4th Per		58 0	2 6	5 4	4 5	5 4
5th Per		74 2	0 7	9 0	0 6	3 5
6th Per		93 2	3 6	11 1	2 4	14 0
7th Per		131 8	4 0	19 2	4 9	19 7
8th Per		116 2	7 3	26 8	4 8	34 1
9th Per		115 2	5 4	42 5	8 8	46 4
10th Per		115 9	7 1	53 3	6 4	71 6

Three flasks in all groups except B, which had 2, values are averages. Culture 2-hr culture *S. paratyphi* A in nutrient broth. Buffer m/2 phosphate pH 6.6. Antibiotic thiolsulfinate, 0.1 mg per ml, glycylcysteine, 0.43 mg per ml, sodium thioglycolate, 0.46 mg per ml, N-acetylcysteine, 0.65 mg per ml.

#### DISCUSSION

On the basis of previously reported work from these laboratories and the data here presented, it is possible to classify antibiotics (using the term in its broadest sense) on the basis of their reactions with thiol compounds. A large number of the antibiotics and the heavy metals are inactivated by thiol, but gramicidin, tyrocidin, streptothricin, and aspergillic acids are not. The antibiotics susceptible to thiol inactivation can further be divided into those causing a bacteriostasis that can be reversed by cysteine and those with which cysteine is without effect on the bacteriostasis. The antibiotics characterized by a thiol-reversible bacteriostasis apparently show little specificity, reacting with most

types of —SH groups. Those with which reversal does not occur are specific, capable of reacting with a specific combination of groups (—SH and adjacent —NH<sub>2</sub>) in proteins. As an example of specificity, it has been observed in our laboratory that whereas urease is not inhibited by penicillin, the enzyme is readily inactivated by the thiolsulfinate, a nonspecific thiol reagent.

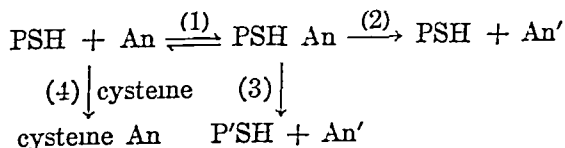
An adequate explanation for the lack of reversibility of bacteriostasis caused by penicillin, streptomycin, and the active principle of *Asarum canadense* is difficult to give. This is largely the result of our lack of knowledge of the chemistry of these antibiotics.

We believe that when a sulfhydryl-reactive antibiotic acts upon an organism to bring about at least bacteriostasis, this effect may be accomplished by the reaction of the antibiotic with biologically essential —SH groups. It is thought that in reversible bacteriostasis the antibiotic reacts only with —SH groups, whereas in irreversible bacteriostasis both —SH and neighboring —NH<sub>2</sub> groups of the protein may be involved. The indirect evidence for this view is to be found in the work of Cavallito and Haskell (1945), who showed that unsaturated lactones reacted in such a manner with aminothiols. Reaction with amino groups probably does not occur with glixoxin, mercuric chloride, and the thiol-sulfinate.

That spacial relations are important in the various reactions of antibiotics is indicated by the fact that whereas  $\beta$ -(dimethylamino)-ethanethiol is very effective in destroying the antibacterial action of penicillin, separation of the thiol and amino groups by one more C atom eliminates the inactivating action. It is entirely possible that within complex proteins, the "geographical position" of the sulfhydryl and amino groups is such that the full reaction, —SH reaction and binding of the protein NH<sub>2</sub>, does not occur. The inability of cysteine to reverse the bacteriostasis of some antibiotics may be due to the combination of antibiotic and protein being so arranged that the cysteine —SH is blocked away from the inactivated enzyme —SH and is prevented thereby from regenerating the latter.

If the action of penicillin is truly bactericidal, as claimed by Chain and Duthie (1945), Bigger (1944), Eriksen (1946), Garrod (1945), and Hobby and Dawson (1944), the inability to reverse the bacteriostasis of penicillin and similar antibiotics is readily explained. Against this explanation is the fact that the concentrations of the antibiotics used in these experiments were bacteriostatic.

The observation that mercuric chloride bacteriostasis can be overcome by thiol compounds is not new. It is of fundamental importance, however, that two antibiotics react in the same manner as does this inorganic antibacterial agent. This points to a similar and basic mode of action of the three antibacterial agents. The concept of antibacterial action may be expressed graphically by the following scheme:



where PSH is the  $-SH$  active enzyme, An is the active antibacterial agent, PSH An is the enzyme-antibiotic reaction product, P'SH is the enzyme that has been dissociated from the antibiotic but altered by the reaction so as to be inert, and An' is the altered antibacterial agent. A bactericidal system could be one in which the reaction between enzyme and antibacterial agent is irreversible (reaction 1) or in which the antibiotic has altered the enzyme so it is inert and is itself altered (reaction 3). Bacteriostasis may be explained by assuming that reaction (1) is reversible, the concentration of antibiotic forcing the reaction to the right, thus leading to suspension of growth, or that reaction (2) occurs.

The resumption of respiration and growth resulting from the treatment of a bacteriostatic system with thiol compounds may be explained in at least two ways. The cysteine may remove the antibacterial agent from the equilibrium reaction (reaction 4). The reversal of mercuric chloride bacteriostasis may be an example of this type of reaction. The thiol compound may displace the antibiotic fragment from the enzyme-antibiotic reaction product with the resulting formation of an inactive, altered antibiotic fragment and the native enzyme (reaction 2). The reaction of the thiolsulfinate and cysteine is believed to be an example of this type.

#### SUMMARY

Bacteriostasis by mercuric chloride, allyl 2-propene-1-thiolsulfinate, and gliotoxin can be reversed by the addition of cysteine to the bacteriostatic system. Bacteriostasis caused by penicillin, streptomycin, the active principle of *Asarum canadense*, or pyocyanin was not reversed by cysteine. In addition to cysteine, glycylcysteine, N-acetylcysteine, and sodium thioglycolate were effective reversing compounds for mercuric chloride, the thiolsulfinate was reversed by all except sodium thioglycolate, whereas only N-acetylcysteine had any effect on the gliotoxin bacteriostasis. Thiol-reactive antibiotics may be classified on the basis of thiol reversibility of the bacteriostasis produced.

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# SOME ALTERATIONS IN CHICKEN ERYTHROCYTES WHICH FOLLOW TREATMENT WITH INFLUENZA AND NEWCASTLE DISEASE VIRUS

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A great deal has been learned about viral hemagglutination since the report by Hirst in 1941 that influenza viruses agglutinate chicken red blood cells. Of special interest have been those observations that suggested that this reaction may serve as a model for the study of viral infections.

In 1942 Hirst reported that PR8 and Lee viruses were eluted from the red blood cells at different rates and that erythrocytes that had adsorbed and fully dissociated from these viruses were no longer capable of adsorbing a detectable amount of fresh homologous or heterologous virus or of agglutinating in their presence. He postulated that this alteration of the erythrocytes was analogous to an enzyme-substrate reaction and suggested that hemagglutination might be an *in vitro* counterpart to natural infection. This suggestion received support from his subsequent work (Hirst, 1943), which indicated that the cells of the respiratory tract resembled red blood cells in their capacity for adsorbing and subsequently dissociating from influenza viruses. Burnet and Bull (1943) in the course of comparative observations on the behavior of freshly isolated influenza strains commented on an apparent parallelism between the susceptibility of erythrocytes from various species to agglutination and the susceptibility of those species to infection. Burnet and his co-workers (1945, 1946) also investigated the agglutinability of red blood cells that had once adsorbed and dissociated from hemagglutinating viruses. Their results, unlike those of Hirst (1942), indicated that there were varying degrees of refractoriness to agglutination depending upon the virus used initially and those used later to test the modified cells. Burnet (1945a,b,c) presented a linear arrangement of these agents—mumps, Newcastle disease, most influenza A strains, influenza B, and swine influenza—and stated that cells treated with any one of them were subsequently refractory to agglutination by the homologous virus and those viruses preceding it in the series, but were still susceptible to agglutination by those following it.

Ziegler and Horsfall (1944) noted certain analogies between interference and the resistance of modified erythrocytes to further agglutination. In their studies of interference in the chick embryo they found that, although PR8 virus causes interference with infection by Lee virus, Lee did not interfere with subsequent infection by large amounts of PR8. This lack of reciprocal interference in the presence of certain viruses is unlike the results reported by Hirst (1942).

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with PR8- and Lee-treated red blood cells, but might be consistent with the observations of Burnet (1945<sup>a,b,c</sup>, Burnet *et al.*, 1946), although he did not report the effect of PR8 in his series. A review of the literature on interference such as is presented by Ziegler and Horsfall (1944) indicates that viruses widely different in antigenicity may cause interference and that this may or may not be reciprocal depending upon the particular agents studied. This has been further documented by the more recent work of Duffy (1944) showing reciprocal interference in the chick embryo with St. Louis encephalitis and Western equine encephalitis viruses, and by that of Lennette and Koprowski (1946) in tissue culture in which yellow fever and West Nile viruses are shown to interfere with infection by PR8 virus, although PR8 does not interfere with infection by the former viruses. The importance of the conditions under which these experiments are performed is shown by a report of Henle and Henle (1945) in which, after using inactivated virus as a first inoculum and an unspecified concentration of active virus as the second, they presented evidence, unlike Ziegler and Horsfall (1944), for reciprocal interference in the chick embryo between PR8 and Lee.

The foregoing findings have many implications for the student of infectious disease. Consequently, it was felt that a reinvestigation with more quantitative techniques of the alterations that take place in erythrocytes following contact with hemagglutinating viruses might reconcile the different results reported by various workers. It was hoped that the results obtained might offer further support for the use of the hemagglutination phenomenon as a tool in the study of the pathogenesis of viral infections. Therefore, the duration and the specificity of the *in vitro* changes in chicken red blood cells following treatment with the influenza viruses, PR8 and Lee, and Newcastle disease virus (NDV) were investigated, and an attempt was made to learn whether similar changes might occur *in vivo* in infected chick embryos. In the present communication it will be shown that the alterations produced in erythrocytes are long-lasting, that the degree of the refractory state for adsorption and agglutination of altered cells varies depending upon the virus employed, and that similar changes do not necessarily occur in infected chick embryos.

#### MATERIALS AND METHODS

**Viruses** The PR8 strain of influenza A virus, the Lee strain of influenza B virus, and a strain of Newcastle disease virus originally recovered by Dr. Beaudette of Rutgers University were used in this study. For convenience they will henceforth be termed PR8, Lee, and NDV, respectively. Pools of allantoic fluids from 12- and 13-day-old chick embryos that had been infected via the allantoic sac were employed as the source of the virus. Between tests these pools were stored at either 4°C or -72°C.

**Cells** Chicken red blood cells were collected in an excess of 2 per cent sodium citrate and washed several times with saline, the desired concentration was prepared from the sediment obtained after centrifugation at 1,500 rpm for 10 minutes.



"Treated" cells were prepared by mixing a suspension of cells with undiluted allantoic fluid. The mixtures were permitted to remain at 37 C and room temperature for varying periods of time before the cells were separated and washed with saline. Washings were repeated many times with intervening periods at 37 C to permit complete elution of absorbed virus. Between procedures the cells were stored at 4 C. Such cells were prepared with PR8, Lee, and NDV, and with normal allantoic fluid and saline. Since several different concentrations of cells were used, more exact details of preparation will be reserved for the experimental section. However, whenever treated cells are referred to in this communication, it is meant to indicate that the cells have been subjected to a procedure such as has just been outlined.

*Saline* An 0.85 per cent solution of sodium chloride containing 0.01 M phosphate buffer at pH 7.2 was used throughout.

*Sera* Specific immune sera were prepared for each of the viruses by injecting rabbits intravenously with 10 ml of a pool of infected allantoic fluid. In preparing NDV and Lee antisera, the rabbits were given a second such injection after 6 weeks—approximately 2 weeks after the last injection serum was collected.

*Hemagglutination tests* The hemagglutination titers of the viruses were obtained by making serial dilutions with saline in a volume of 0.4 ml, adding an equal quantity of a 1.5 per cent red blood cell suspension, and observing the pattern of the sediment after 60 minutes. For PR8 and Lee viruses the reaction was permitted to proceed at room temperature and for NDV usually at 4 C, as previously described (Florman, 1947). In some of the early experiments tests with NDV were conducted at room temperature and read after 30 minutes. The highest dilution of virus giving 2+ agglutination was considered the end point and constituted one hemagglutinating unit of virus. This method was used with fresh red blood cells and with those that had previously been treated with viruses when information was sought regarding their degree of agglutinability.

*Infectivity titrations* The quantity of active virus present in an infected allantoic fluid was determined by titration in chick embryos. Tenfold dilutions were made in 10 per cent normal horse serum broth, and inoculations were carried out by the intra-allantoic route. After 24 hours' incubation of the inoculated embryos the allantoic fluids were collected and tested for hemagglutination. The highest dilution of the inoculum that had produced infection in 50 per cent of the embryos was considered to contain one embryo infective dose (1 EID).

*Antibody titrations* Because tests with NDV are best carried out in the cold and since uniformity was desired, all antibody titrations were done at 4 C by a method previously described for NDV (Florman, 1947). The sera were inactivated at 56 C for 30 minutes, and serial twofold dilutions were prepared in a volume of 0.2 ml. To each there were added 0.2 ml of a virus dilution containing 32 hemagglutinating units. The mixture was shaken and incubated in a 37 C water bath for 60 minutes. After chilling, 0.4 ml of a cold 1.5 per cent chicken cell suspension were added, and the tubes were reshaken and left at 4 C for 60 minutes. At the end of this period the pattern of the sedimented

cells was observed. The lowest dilution of serum permitting 2+ agglutination was considered the end point and constituted one unit of hemagglutination-inhibiting antibody.

#### EXPERIMENTAL RESULTS

*Alterations in agglutinability of virus-treated red blood cells in the presence of fresh virus* In general the published findings of earlier workers were confirmed (Hirst, 1942, Burnet, 1945a,b,c). Chicken red blood cells treated with NDV, Lee, and PR8 showed a modified agglutinability in the presence of fresh homologous or heterologous virus. Cells treated with any one of the three viruses were subsequently inagglutinable in the presence even of large amounts of NDV. However, the degree of alteration of the cells with respect to agglutination with PR8 or Lee was not uniform. As will be shown below, the manner in which the cells were treated and tested, especially with PR8 and Lee, made a difference in the result obtained.

*Persistence of alterations in agglutinability of treated red blood cells* Before any elaborate experiments could be undertaken with altered cells, it was necessary to determine how long after treatment they remained modified. Aliquots of a 3 per cent suspension of freshly collected chicken red blood cells were treated with either NDV, PR8, or Lee by mixing with an equal amount of undiluted, infected allantoic fluid containing the desired virus and by incubating the mixtures at 26 C for a total of 48 hours. After several washings a 1.5 per cent suspension was prepared from each batch of treated cells and a saline control. Then 0.4 ml of these cells were added to 0.4 ml of serial dilutions of fresh preparations of each of the three viruses and hemagglutination end points were determined. At the time the first tests were performed 3 days had elapsed since the cells were treated. Similar tests were subsequently carried out on the tenth and seventeenth days after treatment. In the interim the cells were stored at 4 C as 3 per cent suspensions. In this experiment all the hemagglutination tests were done at room temperature and read after 1 hour except those with NDV, which were read at the end of 30 minutes.

As is shown in table 1, all cells treated in the manner described were inagglutinable by NDV for at least as long as 10 days. Except for those treated with NDV, they were also inagglutinable in the presence of Lee virus for at least as long as 17 days. In the presence of PR8, however, although the NDV- and Lee-treated cells showed diminished agglutinability for as long as 17 days, only the PR8-treated cells were inagglutinable. The appearance with NDV-treated cells of partial agglutinability in the presence of Lee virus on the seventeenth day could not be explained. With other preparations of NDV-treated cells complete loss of agglutinability in the presence of Lee was rarely observed. From this experiment it may be concluded that cells treated with any of these three viruses show altered agglutinability for at least as long as 17 days, and that no essential difference is detectable between results obtained with cells tested on the third and on the tenth days after treatment. In another similar experiment in which only NDV- and Lee-treated cells were studied, such alterations were observed to persist for as long as 21 days.

*Effect of variations in treatment of red blood cells on their agglutinability in the presence of fresh virus* Many difficulties were encountered in obtaining uniform results when treated cells were tested for agglutinability in the presence of fresh virus. It appeared that even though cells were consistently inagglutinable in the presence of large amounts of NDV, the manner of treatment markedly affected the results obtained with PR8 and Lee. Although no attempt was made to investigate this subject completely, two experiments will be presented to illustrate this point.

In one experiment chicken erythrocytes were prepared as a 3 per cent suspension, mixed with equal parts of undiluted, infected allantoic fluid, incubated for

TABLE 1

*Persistence of diminished agglutinability by viruses of red blood cells treated with viruses*

CHICKEN RBC TREATED WITH	VIRUS-INFECTED ALLANTOIC FLUID	HEMAGGLUTINATION TITER† NO. OF DAYS FOLLOWING TREATMENT OF RBC		
		3	10	17
Saline	NDV	1,280	640	
NDV	"	10	10	
Lee	"	10	10	
PR8	"	10	10	
Saline	Lee	1,280	1,280	1,280
NDV	"	10	10	160
Lee	"	10	10	10
PR8	"	10	10	10
Saline	PR8	1,280	640	320
NDV	"	320	160	160
Lee	"	320	160	40
PR8	"	10	10	10

\* Three per cent suspension of RBC added to undiluted allantoic fluid and held at 26 C for 48 hours.

† Reciprocal of dilution giving titration end point.

a total of 17 hours at 37 C, and washed several times before use. These cells will be referred to as "saturated cells." In the second experiment chicken red blood cells were prepared as a 20 per cent suspension, mixed with equal parts of virus, incubated for a total of 6 hours at 37 C, washed several times, and finally stabilized by the addition of a small amount of normal allantoic fluid before use. These cells will be referred to as "unsaturated cells."

Because of the fact that PR8 virus is eluted relatively slowly from red blood cells, it was often difficult to obtain stable suspensions of PR8-treated cells that would not agglutinate spontaneously in saline. Since stable preparations are essential for all tests of comparative agglutinability, very long incubation periods and many washings were usually required. Fortunately it was observed that by the addition of small amounts (e g, 12 per cent) of normal allantoic fluid, which contains an hemagglutination inhibitor, as was indicated above, the period of preparation could be greatly shortened.

From the tenth to the twelfth days after the preparation of the saturated and unsaturated cells was started, hemagglutination tests were conducted. Dilutions of NDV, Lee, and PR8 viruses were made in saline so that each dilution contained a known number of hemagglutinating units. In the first experiment these ranged from 32 to 256 units and in the second from 1 to 512 units. To 0.4 ml of each virus dilution there were added 0.4 ml of a 1.5 per cent suspension of virus-treated and control chicken cells. Tests in which NDV was used as the agglutinating agent were performed at 4°C, the others were done at room temperature. At the end of 1 hour the degree of agglutination in each tube was noted and compared.

The results are shown in table 2. It is seen that both the saturated and the unsaturated cells were equally magglutinable in the presence of NDV. However, the unsaturated cells were apparently less altered, especially by NDV and PR8.

TABLE 2  
*Agglutinability by viruses of red blood cells variously treated with viruses*

CHICKEN RBC TREAT ED WITH	TREATMENT	RESULTS* OF HEMAGGLUTINATION TESTS WITH INDICATED NUMBER OF HEMAGGLU- TINATING UNITS OF VARIOUS VIRUSES																												
		NDV										Lee										PR8								
		512	256	128	64	32	16	8	4	2	1	512	256	128	64	32	16	8	4	2	1	256	128	64	32	16	8	4	2	1
Saline	Saturated 3% suspension of RBC treated at 37 C for 17 hr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
NDV		4	4	4	4	—	—	—	—	—	—	4	4	4	4	—	—	—	—	—	—	4	4	4	4	—	—	—	—	—
Lee		0	0	0	0	0	—	—	—	—	—	3	3	2	1	—	—	—	—	—	—	4	4	4	4	—	—	—	—	—
PR8		0	0	0	0	0	—	—	—	—	—	0	0	0	0	—	—	—	—	—	—	4	4	4	3	—	—	—	—	—
NAF†		0	0	0	0	0	—	—	—	—	—	0	0	0	0	—	—	—	—	—	—	3	2	0	0	—	—	—	—	—
		4	—	—	4	—	—	—	—	—	4	—	—	4	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
Saline	Unsaturated 20% suspen sion of RBC treated at 37 C for 16 hr	4	4	4	4	4	4	4	4	3	2	4	4	4	4	4	4	4	4	3	4	4	4	4	3	3	3	2	2	±
NDV		0	0	0	0	0	0	0	0	0	0	4	4	4	4	3	2	2	2	2	4	4	4	4	3	3	2	2	±	0
Lee		0	0	0	0	0	0	0	0	0	0	±	±	0	0	0	0	0	0	0	4	4	4	4	3	2	1	0	0	±
PR8		0	0	0	0	0	0	0	0	0	0	4	4	4	3	2	0	0	0	0	4	4	4	4	3	3	3	2	±	±
NAF		4	4	4	4	4	4	4	3	3	2	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	3	2	±

\* 4 = complete agglutination, 3, 2, and 1 = partial agglutination (2 is considered end point), 0 = no agglutination.  
† NAF = normal allantoic fluid.

In the presence of Lee and PR8 these cells show less loss of agglutinability than did the saturated cells, although in both experiments the differences from the controls were in the same direction. The differences between the reactivity of Lee-treated cells with Lee virus were minimal. In neither instance could definite agglutinability be demonstrated even in the presence of 256 hemagglutinating units of virus.

If the first experiment is considered alone, it would appear that PR8 virus was most effective in modifying the agglutinability of chicken red blood cells, followed closely by Lee and then by NDV. However, if the second experiment is considered alone, the order is slightly changed. Lee is the most effective in altering the reactivity of the cells, followed by PR8 and then NDV. If these results are considered in terms of exhausting the receptors of the erythrocytes for these viruses, as has been suggested, they offer evidence to support Burnet (1945b) that the receptor for NDV is most readily removed. The receptor for

Lee would seem to be more quickly exhausted than that for PR8, although when prolonged treatment is permitted, that for PR8 virus appears to be slightly more effectively removed

Table 2 also shows the advantage of testing treated cells against more than one dilution of virus. In the first experiment quite different results would have been obtained if a single dilution of 256 or of 64 units of PR8 had been used, and similarly in the second experiment, if just 64 or 16 units of Lee had been employed

Neither of these experiments shows the complete reciprocal loss of agglutinability in the presence of PR8 and Lee by PR8- and Lee-treated cells reported by Hirst (1942). However, the results of tests with saturated cells and 64' or fewer hemagglutinating units in the first experiment do approach this and suggest that the method of treatment be considered in all its details in any discussion of alteration of agglutinability of treated erythrocytes

*Alterations in adsorptive capacity of treated red blood cells* From his studies with PR8 and Lee, Hirst (1942) reported that changes in agglutinability of treated cells are paralleled by changes in their capacity to adsorb fresh virus. A number of experiments were, therefore, conducted with NDV, PR8, and Lee viruses in order to learn whether changes could be detected in the adsorptive capacity of treated cells comparable to the modifications in their agglutinability described previously

Appropriate mixtures of red cells and viruses were prepared. After a designated time the amount of virus adsorbed by the cells as reflected in a decrease in the hemagglutinating titer of the supernatant was determined. In a few experiments the amount of virus that could be subsequently eluted from the sedimented cells was also measured

Experiments were carried out with the same saturated and unsaturated chicken red cells that were used in the agglutination experiments presented in table 2. However, the former cells were 12 days old at the time of the test and the latter only 2 and 3 days old. Since it was not necessary to have completely stabilized cells, the unsaturated cells were merely suspended in saline. Adsorption with NDV was permitted to take place for 5 to 10 minutes at 4 C, with Lee for 10 minutes at room temperature, and with PR8 for 40 minutes at room temperature. These intervals were selected after preliminary experiments with untreated red cells had shown them to be satisfactory for each of the viruses. After the sediment was washed, virus was permitted to elute into fresh saline at 37 C. In the experiment with saturated cells the detection of eluted virus was not begun until after the cells and virus had been together at room temperature for 1 hour, in the experiment with unsaturated cells this was begun immediately after the previously indicated intervals. In the former experiment the amount of virus adsorbed by a 3 per cent cell suspension was studied, and in the latter the amount adsorbed by a 10 per cent suspension was determined

The results are summarized in table 3. The first experiment was with saturated cells. It was found that none of the treated cells adsorbed any NDV that could be detected either by reduction in titer of the supernatant or by elution

from the sedimented cells. Although none of the virus-treated cells reduced the titer of the Lee supernatant, the NDV- and Lee-treated cells apparently adsorbed some of this virus for it was subsequently eluted from them. The greater sensitivity of elution as an index of adsorption capacity is even better illustrated with these cells and PR8. In no instance with virus-treated cells was the titer of the supernatant significantly reduced, yet all three of the types of virus-treated cells later showed elution of some virus. The fact that a 50 per cent reduction in virus concentration must take place before it is reflected by a single tube change in the former test no doubt accounts for the foregoing findings. The results of the second experiment in which more concentrated

TABLE 3  
*Adsorption of viruses by red blood cells variously treated with viruses*

TREATED WITH	CHICKEN RBC TREATMENT	CONCENTRATION USED FOR ADSORPTION	HEMAGGLUTINATION TITERS* OF VIRUS REMAINING IN THE SUPERNATANT AND ELUTED FROM THE RBC SEDIMENT FOLLOWING ADSORPTION OF INDICATED VIRUSES					
			NDV		Lee		PR8	
			Super-natant	Eluate from RBC	Super-natant	Eluate from RBC	Super-natant	Eluate from RBC
(Control—no RBC)	Saturated 3% suspension of RBC treated at 37 C for 17 hr	3	640	—	1,280	—	320	—
Saline			160	64	320	16	<20	512
NDV			640	<4	2,560	8	160	16
Lee			1,280	<4	2,560	4	640	32
PR8			640	<4	1,280	<4	160	32
(Control—no RBC)	Unsaturated 20% suspension of RBC treated at 37 C for 6 hr	10	128	—	1,024	—	384	—
Saline			48	16	32	>128	<4	38
NDV			128	<2	128	96	32	54
Lee			128	<2	1,024	2	128	18
PR8			128	<2	1,024	4	128	13
NAF			48	16	32	>128	<4	35

\* Reciprocal of dilution giving titration end point

although unsaturated cells were used differed from the first chiefly in quantitative aspects. In those instances in which virus was adsorbed, it was usually reflected by both a drop in titer of the supernatant and a greater elution of virus from the sedimented cells. This might have been because a more concentrated cell suspension was used, and hence more cells were available for adsorbing virus, or because the less completely treated cells were less modified, or because of a combination of both factors. It would seem that the most reliable results would be obtained if saturated cells and more concentrated cell suspensions were used.

A comparison of the results presented in tables 2 and 3 in general confirms Hurst's impression that there are parallel changes in the agglutinability and absorptive capacity of treated cells. This correlation is especially good when

NDV is used as the test agent, somewhat less so when PR8 is the virus, and only fair in the presence of Lee. Even though PR8-treated cells were agglutinated by 32 units of Lee in the second experiment, the eluate gave the only evidence that there had been any adsorption of virus. Conversely, the Lee-treated cells that were inagglutinable by 256 units of Lee apparently did adsorb some virus, as could subsequently be detected by elution. These findings indicate once again that the alterations produced in red blood cells are to a degree specific for the virus used. Those induced by NDV and PR8 are most apparent when NDV is used as the test agent, less so if Lee is the virus to be adsorbed, and least in the presence of PR8. In addition, in arranging these three viruses in order of ascending degrees of effectiveness in modifying the adsorptive capacity of chicken erythrocytes the order is also NDV, Lee, and PR8 although the difference between PR8 and Lee is very slight.

*Attempt to correlate alterations in treated red blood cells with those in infected chick embryos.* The superficial resemblance of the induction of refractory red blood cells to interference has been noted by others (Ziegler and Horsfall, 1944, Stone, 1947). It was hoped that the present investigation might offer support for the use of hemagglutination as a model for the study of viral infections. The results of experiments with virus-treated red blood cells such as those that have been presented above suggested a direct approach to this question. In all of the experiments described erythrocytes treated with PR8 were subsequently unable to adsorb or to be agglutinated by NDV, whereas cells treated with NDV were still able to adsorb and to be agglutinated by PR8 almost as well as were the controls. Therefore, if hemagglutination were a satisfactory model of virus infection, changes similar to these might be expected to occur in chick embryos following infection with these viruses. Chick embryos infected by PR8 should be resistant to infection by NDV, whereas embryos infected by NDV should still be susceptible to PR8.

Before this possibility could be examined, a method had to be devised that would permit the accurate detection of relatively small amounts of one of these viruses in the presence of the other. After a number of trials with allantoic fluids containing a mixture of two viruses, the following procedure was found to be sufficiently sensitive for this purpose. Serial dilutions of a fluid were made in triplicate in 0.2-ml quantities. There were then added to each tube of one series 0.2 ml of a 1:20 to 1:100 dilution of immune rabbit serum containing 32 antibody units against one of the viruses, to each tube of the second series the same amount of rabbit antiserum against the second virus, and to the third series a dilution of normal rabbit serum equal to that of the more concentrated of the other two sera. It was, however, necessary to select sera that, when diluted as just indicated, would show no nonspecific inhibition or chicken red blood cell agglutinins. The subsequent procedures were similar to those employed in titrating antisera. The mixtures were shaken, incubated at 37 C for 60 minutes, and chilled, after which 0.4 ml of a cold 1.5 per cent chicken red blood cell suspension were added. The end point in the presence of each of these sera was the highest dilution of a fluid giving a 2+ agglutination. As controls, allantoic

fluids that contained only one of the two viruses were similarly examined. With each fluid the titers obtained in the presence of normal rabbit serum were compared with those obtained in the presence of each of the two antisera. Reduction of the hemagglutination titer by an immune serum is a measure of the amount of homologous virus present. However, this reduction may be obscured and made to appear less if heterologous virus is also present in the mixture. Therefore, the degree of reduction in titer of a control fluid that contained only

TABLE 4  
*Serologic analysis of mixtures of allantoic fluids containing two viruses*

MIXTURE OF INFECTED ALLANTOIC FLUID		RABBIT SERUM*	RESULTS† OF HEMAGGLUTINATION INHIBITION TESTS FINAL DILUTION OF ALLANTOIC FLUID MIXTURES											INHIBITION END POINT
PR8	Lee		4	8	16	32	64	128	256	512	1 024	2 048		
<i>ml</i>	<i>ml</i>													
1 0	0 0	Normal	4	4	4	4	4	4	3	2	0	0	512	
		Anti-PR8	4	2	0	0	0	0	0	0	0	0	8	
		Anti-Lee	4	4	4	4	4	4	2	0	0	0	256	
0 0	1 0	Normal	4	4	4	4	4	4	3	2	0	0	512	
		Anti-PR8	4	4	4	4	4	4	3	1	0	0	256	
		Anti Lee	3	0	0	0	0	0	0	0	0	0	4	
0 5	0 5	Normal	4	4	4	4	4	4	3	2	1	0	512	
		Anti-PR8	4	4	4	4	4	3	0	0	0	0	128	
		Anti-Lee	4	4	4	4	4	3	0	0	0	0	128	
0 95	0 05	Normal	4	4	4	4	4	4	3	3	1	0	512	
		Anti-PR8	4	4	4	2	0	0	0	0	0	0	32	
		Anti-Lee	4	4	4	4	4	4	2	0	0	0	256	
0 05	0 95	Normal	4	4	4	4	4	4	3	2	0	0	512	
		Anti-PR8	4	4	4	4	4	4	3	0	0	0	256	
		Anti-Lee	3	3	3	0	0	0	0	0	0	0	16	

\* Constant amount of serum was used, final dilutions ranged from 1/20 to 1/100, corresponding to approximately 32 hemagglutination inhibition units.

† 4 = complete agglutination, 3, 2, and 1 = partial agglutination (2 is considered end point), 0 = no agglutination.

one virus was first determined and then compared with the degree of reduction demonstrable with the same sera and a fluid suspected of being a mixture. If in an immune serum the decrease in titer of the unknown was less than that exhibited with the control fluid, heterologous virus was assumed to be present. With this method it was possible with considerable accuracy to determine the composition of mixtures of any two of the group of PR8, Lee, and NDV, as is done, for example, in table 4 with PR8 and Lee. It was also possible to undertake the study of the changes in susceptibility of chick embryos that follow infection.



Several experiments were performed in which 11-day-old chick embryos were given two intra-allantoic inoculations 24 hours apart. These were given through a small shell hole that had been made directly over the embryo. Reinoculations were done through a paraffin seal over the site of the first inoculation. The volume of the first inoculum was 0.1 ml and of the second 0.5 ml. The inocula

TABLE 5

*Serologic analysis of representative allantoic fluids from chick embryos inoculated with two viruses*

INOCULA (INTRA ALLANTOIC)					RABBIT SERUM*	HEMAGGLUTINATION INHIBITION END POINTS†	VIRUS DEMONSTRATED	
First		Interval	Second				PR8	NDV
0.1 ml	E I D		0.5 ml	E I D				
NDV	10 <sup>5.0</sup>	<i>hours</i> 24	Broth	—	Normal Anti-PR8 Anti-NDV	512 512 16	0	+
NDV	10 <sup>5.0</sup>	24	PR8	10 <sup>6.7</sup>	Normal Anti-PR8 Anti-NDV	256 256 8	0	+
PR8	10 <sup>4.0</sup>	24	Broth	—	Normal Anti-PR8 Anti-NDV	512 8 256	+	0
PR8	10 <sup>4.0</sup>	24	NDV	10 <sup>6.7</sup>	Normal Anti-PR8 Anti-NDV	512 16 256	+	0
Broth	—	24	NDV	10 <sup>6.7</sup>	Normal Anti-PR8 Anti-NDV	512 512 16	0	+
Broth	—	24	PR8	10 <sup>5.7</sup>	Normal Anti-PR8 Anti-NDV	1,024 16 512	+	0

\* Constant amount of serum was used, final dilution ranged from 1/20 to 1/100, corresponding to approximately 32 hemagglutination-inhibition units

† Reciprocal of dilution giving titration end point

contained large amounts of virus in order to simulate the conditions under which the red blood cells were treated. They contained approximately 10,000 and 500,000 E I D, respectively, of PR8, and 100,000 and 5,000,000 E I D, respectively, of NDV. Twenty-four hours after the second inoculation the eggs were removed from the incubator to the refrigerator and chilled before the allantoic fluids were collected. These fluids were analyzed for virus content by the method described above.

The results presented in table 5 are representative of those obtained in numerous double infection experiments. The susceptibility of the embryo was so altered by whichever virus first infected it that the secondary introduction of very large amounts of heterologous virus failed to induce infection. Unlike what occurred *in vitro* with red cells, there was no evidence that the PR8 virus was able to infect cells that had been previously infected by NDV. Indeed, a total of 11 embryos were given a preliminary inoculation of 100,000 E I D of NDV, followed after 24 hours by 500,000 E I D of PR8. When the allantoic fluids were removed 24 hours later, NDV was the only virus that could be detected in any of them. Yet the controls indicated that this amount of PR8 produced maximal infection in normal chick embryos.

Ziegler and Horsfall (1944), by using very large inocula comparable to those in the present experiments, were able to show that, although infection of chick embryos by PR8 uniformly interfered with subsequent infection by Lee, a primary infection by Lee was not always able to prevent later infection by large amounts of PR8. Such results would seem to be consistent with what one might have predicted from agglutination experiments with treated erythrocytes such as are given in table 2. Consequently, the failure to show a similar correlation between the results with PR8 and NDV in the foregoing experiments appears significant. It indicates that different mechanisms are probably involved in inducing a refractory state in red blood cells and in modifying cells of chick embryos so that a second infection is blocked. It also emphasizes an inadequacy of the hemagglutination phenomenon as a model of virus infections.

#### DISCUSSION

The nature of the characteristic changes that are produced in erythrocytes by hemagglutinating viruses is still not known. It has been suggested that they result from "exhaustion of the receptors" of the cells (Hirst, 1942). Indeed, Burnet *et al.* (1946) have presented evidence that a lecithinase, if permitted to act for varying periods of time, may modify the reactivity of red blood cells in a linear fashion somewhat similar to the hemagglutinating viruses. From this it was implied, as by Hirst (1942) previously, that the exhaustion may be enzymatic in nature. Bovarnick and de Burgh (1947) have recently presented evidence that the receptors of erythrocytes for the several hemagglutinating viruses may be different. They prepared a lipid extract from sheep red blood cells that prevented agglutination of human and chicken erythrocytes by mumps virus but not by PR8, whereas a similar extract from human red blood cells prevented agglutination of these cells by both mumps and PR8. Although the evidence is not yet complete, it is very suggestive that in hemagglutination there is a reaction between a component in or on the red blood cell surface and the virus that results in a permanent modification of the cell surface. This modification appears to be specific for the hemagglutinating virus.

The changes in the reactivity of virus-treated red blood cells are so striking that it was natural to inquire whether similar alterations might occur in tissue cells after infection, even though the many differences between these two types

of cells and in the procedures are quite obvious. A simple experiment with which to seek an answer to this question was suggested by the observation that cells treated with PR8 were subsequently unable to adsorb or to be agglutinated by NDV, whereas cells treated with NDV subsequently absorbed and were agglutinated by PR8 virus to essentially the same degree as were controls. Therefore, chick embryos were infected first with PR8 or NDV and subsequently challenged with large amounts of the heterologous virus. Unlike the virus-treated red blood cells, they manifested no selective blocking of the second virus. Whichever virus was introduced first prevented infection by the second.

The foregoing findings are consistent with the results of experiments by Henle and Henle (1947) that also indicate a difference between the capacity of a virus to render red blood cells refractory to agglutination and its capacity to block infection. These authors treated influenza viruses with ultraviolet light for varying periods of time and were able to demonstrate that the property of blocking infection could be destroyed much more quickly than the property of rendering red blood cells refractory to agglutination.

#### SUMMARY

Alterations characteristic of the virus employed take place in chicken red blood cells following treatment with Newcastle disease virus (NDV) and the two influenza viruses, PR8 and Lee. These changes are reflected in parallel losses in the adsorptive capacity and agglutinability of treated cells for fresh homologous and heterologous virus. These modifications in red blood cells may persist for at least as long as 21 days. The extent of the alterations is directly proportional to the intensity of viral treatment. When a 3 per cent suspension of chicken erythrocytes is treated for 17 hours at 37 C with NDV, Lee, or PR8 viruses, the most marked changes are produced by PR8, slightly less striking ones follow treatment with Lee, and the least modifications follow treatment with NDV. If the cells are less thoroughly treated, the order is Lee, PR8, and NDV. Further evidence is presented that the hemagglutination phenomenon is an inadequate model for the study of viral infections. The modifications in erythrocytes that follow treatment with NDV are not correlated with changes that follow infection of chick embryos by this virus. Although red blood cells that have been treated with NDV are still capable of adsorbing and being agglutinated by PR8, chick embryos that have been infected by NDV are after 24 hours no longer susceptible to infection with as much as 500,000 embryo infective doses of PR8.

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# THE CONVERSION OF SUCROSE TO A POLYSACCHARIDE OF THE STARCH-GLYCOGEN CLASS BY *NEISSERIA* FROM THE PHARYNX

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In a recent preliminary note (Hehrl and Hamilton, 1946), we reported that certain bacteria of the *Neisseria* genus convert sucrose (but not other common sugars) to a polysaccharide resembling amylopectin or glycogen and that they can accomplish the synthesis without utilizing glucose-1-phosphate as an intermediate substance. This bacterial synthesis is of special interest because substances belonging to the starch or glycogen class have been generally assumed (see, for example, Cori, 1945; Hassid, 1946) to require glucose-1-phosphate as the substrate. The experiments of the present paper deal with the occurrence and biological properties of the bacteria that bring about this synthesis and with some of the conditions that influence the production of the polysaccharide.

## OCCURRENCE IN HUMAN THROATS

The results of the following experiment illustrate the common occurrence in human throats of strains of *Neisseria* that form amylopectinlike polysaccharide from sucrose. Swabs of the posterior pharyngeal wall of 12 young adults were pressed out in 1-ml amounts of broth. Two loops of each suspension were spread in exactly the same way over the surface of two plates of 5 per cent sucrose gelatin agar, one containing no penicillin and the other containing 0.1 unit per ml of commercial crystalline penicillin G; the sucrose agar was the same as described by Niven, Smiley, and Sherman (1941a). The plates were incubated aerobically at 37 C for 2 days and then examined both for total number of colonies and for the number that darkened on treatment with a solution containing 0.2 per cent iodine and 0.4 per cent potassium iodide.

The chief point in table 1 is that bacteria that produce colonies giving a dark maroon color with iodine were obtained from the throats of each of the 12 persons examined. In the majority of instances iodophilic colonies were frequent on the sucrose agar plates containing penicillin and outnumbered the other colonies. On the medium without penicillin, except for two persons (Kn and He), colonies reacting with iodine were much less frequent and were greatly outnumbered by the nonreacting colonies. This difference between the plates that contained penicillin and those that did not was probably due to the suppression by the penicillin of streptococci, which have been observed by others to inhibit the development of colonies of *Neisseria* on agar plate cultures of material from the throat. The penicillin can be considered to permit the development of the neisseriae, and the sucrose to furnish the substrate for produc-

tion of the polysaccharide by which the colonies of these neisseriae are recognized. The roles of the sucrose and penicillin are illustrated in figure 1. Equal amounts of a suspension of material from the posterior pharynx of a healthy person were inoculated on plates of four different media. *a* and *b*, the 5 per cent sucrose agar

TABLE 1  
*Occurrence in human throats of bacteria that form starchlike material from sucrose*

PLATING MEDIUM	IODINE REACTION OF THE COLONIES	NUMBER OF COLONIES ON THE PLATES INOCULATED WITH MATERIAL FROM 12 PERSONS											
		Br	Ca	Ep	Pa	Ha	Mu	Sa	Ma	Si	Wi	Kn	He
With penicillin	Dark maroon	250	200	10	11	7	19	18	32	12	1	150	120
	No color	0	0	1	3	0	4	12	28	13	60	100	80
Without penicillin	Dark maroon	30	20	4	4	0	2	1	0	3	0	150	140
	No color	270	400	100	150	300	100	150	300	250	100	250	340

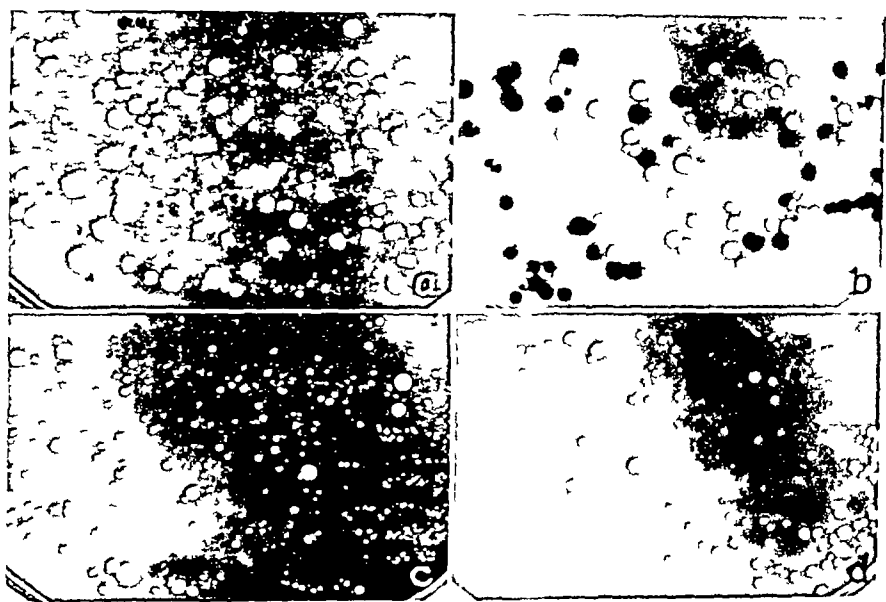


FIG 1 IODINE REACTIONS OF BACTERIAL COLONIES OBTAINED FROM MATERIAL FROM THE PHARYNX OF A HEALTHY PERSON

(*a*) Sucrose agar, (*b*) sucrose agar plus penicillin, (*c*) glucose agar, (*d*) glucose agar plus penicillin

used in the experiment of table 1, with and without penicillin, *c* and *d*, the same basic agar medium but with glucose instead of sucrose, with and without penicillin. After 2 days' incubation at 37 C all four plates were treated with iodine solution. A much higher proportion of iodine-coloring colonies occurred on the sucrose plate containing penicillin than on the sucrose plate without penicillin; none of the colonies on either of the glucose plates darkened with iodine because

those mediums lacked the substrate (sucrose) required for production of the polysaccharide

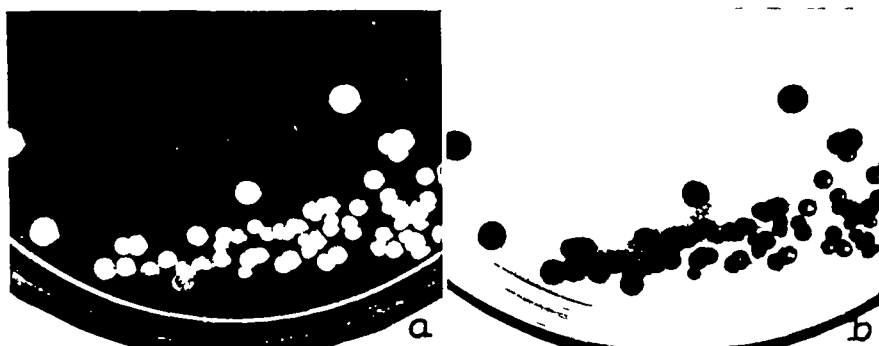


FIG. 2. MUCOID COLONIES OF A STRAIN OF POLYSACCHARIDE FORMING NEISSERIA ON 5 PER CENT SUCROSE GELATIN AGAR  
(a) Untreated, (b) treated with iodine

TABLE 2

*Descriptive features of 39 strains of Neisseria that produce a polysaccharide of the starch glycogen class from sucrose*

<i>Source</i>	pharynx and nasopharynx of healthy people
<i>Morphology</i>	cocci, usually biscuit shaped pairs, predominantly gram-negative
<i>Nutrition</i>	grow well on peptone media without blood or ascitic fluid
<i>Catalase</i>	hydrogen peroxide decomposed, usually rapidly
<i>Oxidase</i>	colonies darken on treatment with dimethyl-paraphenylene diamine
<i>Pigment</i>	faint yellow colonies on glucose agar
<i>Temperature for growth</i>	grow well at 37 C, also grow at 25 C
<i>Oxygen tension</i>	aerobic, grow poorly if at all anaerobically, often form a surface ring or pellicle on fluid media
<i>Resistance to penicillin</i>	develop rapidly in presence of 0.25 unit per ml
<i>Carbohydrate fermentation</i>	acid from glucose, fructose, maltose, sucrose, and raffinose Acid from mannitol by 36 of the 39 strains, acid from lactose, xylose and arabinose by 25 of the strains
<i>Polysaccharide formation</i>	iodine darkening material produced by cultures grown with sucrose, but not when grown with glucose, fructose, glucose plus fructose, maltose, raffinose, mannitol, lactose, xylose, or arabinose
<i>Colony appearance</i>	flat, smooth surfaced colonies on glucose agar Colonies on sucrose agar of about half the strains dome shaped and pearly mucoid (see figure 2), those of other strains not different in gross appearance from colonies on glucose agar

#### GENERAL PROPERTIES OF THE BACTERIA

Thirty-nine strains, isolated from the throats of 17 healthy people and known from preliminary observations to produce iodophilic material when grown in the presence of sucrose, were studied in respect to some of the taxonomic properties commonly used in the description of *Neisseria*, and also in respect to colonies on sucrose and glucose agar, and polysaccharide formation from various sugars. A summary of the results is in table 2.

*Taxonomic properties* Tests for catalase, oxidase, and pigment were made on cultures grown on 0.5 per cent glucose agar. Ability to grow aerobically at 37 C and at 25 C, and in a hydrogen jar at 37 C, was tested in meat infusion broth containing added glucose and yeast extract. Penicillin sensitiveness was tested by adding minute inocula to broth containing 0.25 units per ml of crystalline penicillin G. Tests for acid production were made on agar slants containing

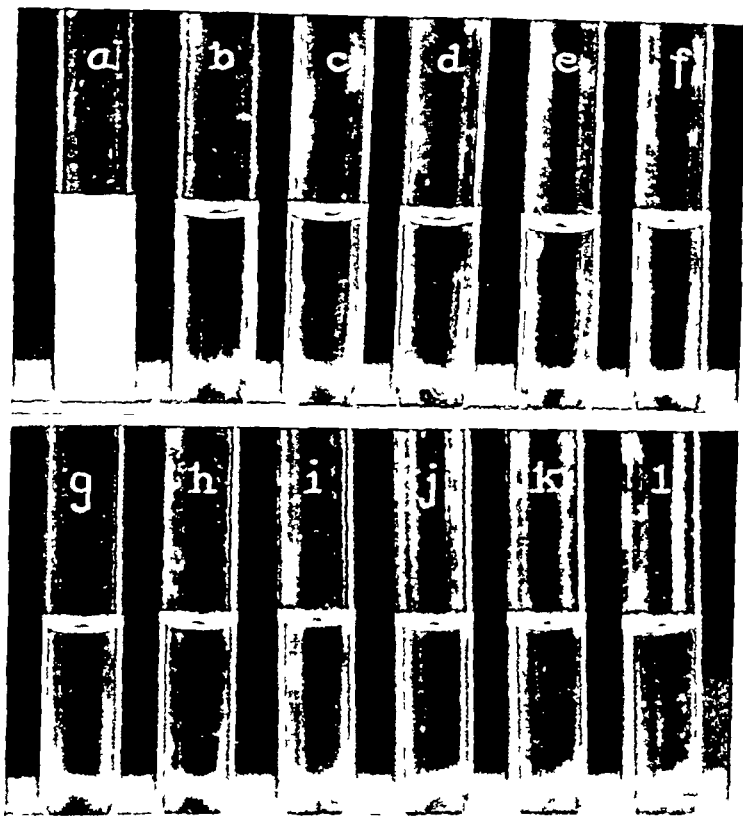


FIG. 3. CULTURE FLUIDS OF A STRAIN OF *NEISSERIA PERFLAVA* GROWN WITH DIFFERENT SUGARS.

(a) sucrose, (b) glucose + fructose, (c) glucose, (d)  $\alpha$  methyl glucoside, (e) maltose, (f) lactose, (g) trehalose, (h) raffinose, (i) fructose, (j) galactose, (k) xylose, (l) arabinose.

0.1 M concentrations of various sugars, one part of a sterile 1.0 M solution of the sugar was added aseptically to nine parts of sterilized base comprising 1 per cent tryptose, 0.5 per cent Difco yeast extract, 0.5 per cent NaCl, 0.2 per cent  $\text{Na}_2\text{HPO}_4$ , 0.0025 per cent phenol red, and 1.8 per cent agar, the cultures were observed over a period of 6 days.

In general, the properties of the strains of the present collection (table 2) agree well with those described for *Neisseria postflava* in *Bergey's Manual* (1939), and for purposes of brevity that name is used in this paper for the present *Neisseria*. Eleven strains of other varieties of *Neisseria* belonging to the "pharyngeal group" but differing in sugar fermentation and some other properties from the



definition proposed for *N. parflava* (including some that could be classified as *N. catarrhalis*, *N. sicca*, and *N. flava*) were tested and found to lack the capacity of forming starchlike material from sucrose. The eight strains of *N. gonorrhoeae* and three of *N. meningitidis* that were tested also proved negative.

**Substrate specificity.** The same sugar agar slant cultures that were utilized to determine capacity for acid production were tested for polysaccharide production by the addition of a few drops of 0.2 per cent iodine solution to the slant surfaces. All strains gave a dark mucron color in the sucrose agar cultures but not in the cultures containing any of the other eight sugars. The marked influence of the presence of sucrose in the culture medium is important because it emphasizes the distinction between the production of material of the starch-glycogen class by cultures of *Neisseria* from the long-recognized phenomenon of

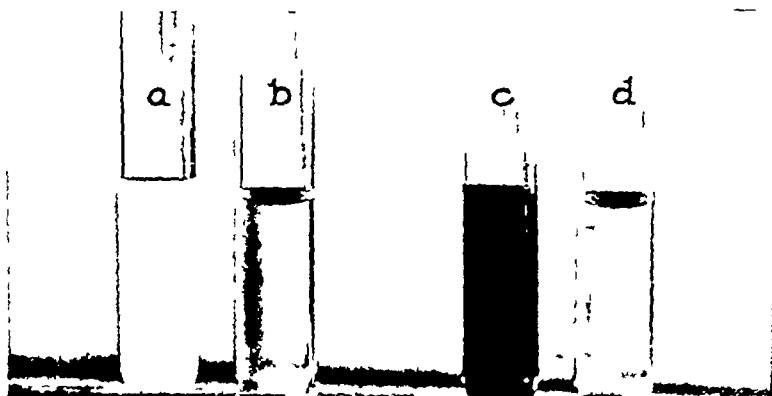


FIG. 4. EFFECT OF SALIVA ON SUCROSE BROTH CULTURE OF A STRAIN OF *NEISSERIA PARFLAVA*.

(a) Untreated fluid, showing pronounced opalescence, (b) same fluid, treated with saliva, showing loss of opalescence, (c) dark color of original fluid on addition of iodine, (d) loss of iodine coloring capacity of fluid treated with saliva.

production of granules of "glycogen" or of "reserve carbohydrate" by various bacteria and fungi, which has never been related to any particular substrate. The same influence of sucrose is evident in broth cultures, as shown in figure 3, which illustrates the pronounced difference in respect to opalescence between the culture fluids of a typical strain grown with 0.1 M concentrations of sucrose and of 11 other common sugars.

#### FORMATION OF POLYSACCHARIDE IN SUCROSE BROTH CULTURES

The following experiment was made to compare the action on sucrose of the collection of neisseriae described in table 1 with the action on sucrose of *Neisseria* strain 19-34 from which we have obtained a chemically identified amylopectin-like polysaccharide (Hehre and Hamilton, 1946), and to compare their action also with that of bacteria known to form other polymer products (dextran and levan) from sucrose. The dextran former employed was *Streptococcus* group H, F90A (Hehre and Neill, 1946), the levan former was *Streptococcus salivarius*,

S20B (Niven, Smiley, and Sherman, 1941b) Duplicate cultures of all the strains were made—one series in sucrose broth and another series in the same basic broth but with glucose in place of sucrose. The latter was included as an aid in the interpretation of the results obtained with the sucrose cultures.

The broth comprised 1.0 per cent tryptose, 0.5 per cent NaCl, 0.2 per cent  $\text{Na}_2\text{HPO}_4$ , and either 5.0 per cent sucrose or 5.0 per cent glucose, and was tubed in amounts to give a depth of 3 cm. (The production of polysaccharide by *Neisseria* cultures growing in 5 per cent sucrose broth is greatly impaired if the depth of the medium is much greater than 3 cm or if the culture is disturbed during growth.) The sucrose was a selected sample of beet sugar that was relatively free of material precipitable by 65 per cent alcohol, it was utilized in place of reagent sucrose because most lots of the latter (Neill, Hehre, Sugg, and Jaffe, 1939) contain material that would interfere with some of the chemical and serological tests included in the experiment. The glucose was sterilized separately and added aseptically. Tubes of both media were inoculated with the equivalent of 0.001 ml of a blood broth culture and were incubated for 6 days at 37°C.

The cultures were centrifuged at 1,500 rpm for 40 minutes and the supernatant fluids separated from the bacterial sediments. The bacterial cells were examined for capacity to blacken on addition of 0.2 per cent iodine reagent (test 1), the supernatant fluids were examined for opalescence (test 2), capacity to give a visible precipitate with 1.2 volumes of 95 per cent alcohol when diluted 1:10 with 5 per cent sodium acetate (test 3), kind and intensity of color produced by treatment with iodine (test 4), capacity to give serological precipitation with dextran- and levan-reactive antisera (test 5), and the presence of free reducing sugars (test 6).

Test 4, which was made to get some idea of the relative amount and type of polysaccharide present in the culture fluids, was performed by treating duplicate samples of 0.5 ml of supernatant fluid with 4.5 ml of 0.1 acetate buffer (pH 5.6) and 0.1 ml of solution containing 1 per cent iodine and 2 per cent potassium iodide. Each mixture was placed in a standardized 1-cm colorimeter tube and its light absorption measured immediately in a Klett-Summerson photoelectric colorimeter. A green filter (no. 54, of approximate spectral range 500 to 570  $m\mu$ ) was used for one mixture, a red filter (no. 66, range 640 to 700  $m\mu$ ) for the other. The light absorption of the buffer iodine solution was used to set the zero point in each case. In test 5 type II pneumococcus antiserum was used to detect dextrans, and antiserum of a levan-forming bacillus to detect levans, the procedure was the same as that described by Hehre and Neill (1946). In test 6 the presence of free reducing sugars in the bacterial cultures was judged by heating 0.5 ml of the fluid with 2.0 ml of Benedict's qualitative reagent at 100°C for 5 minutes.

In the cultures containing sucrose, all the strains of *N. perflava* formed some material of the starch-glycogen class as indicated (table 3) by the darkening of the bacterial sediments and of the supernatant fluids on treatment with iodine. With the majority of the strains a considerable amount was extracellular, the

fluids freed from the bacterial cells showed pronounced opalescence, abundant precipitation with 12 volume of alcohol, and a strong color with iodine. In respect to the quality of color with iodine, the extracellular product formed by all the *Neisseria* strains was similar to that formed by the strain (19-34) from which a polysaccharide chemically like amylopectin or glycogen was isolated (Hehre and Hamilton, 1946). That is, the sucrose culture fluids of all the *Neisseria* gave a maroon color and showed much less light absorption in the red than in the green regions.

The opalescence, precipitation with alcohol and accumulation of reducing sugars in the sucrose cultures, and the absence of the opalescence and precipitation in the glucose cultures, which was shown by nearly all the *Neisseria* strains,

TABLE 3

Formation of a polysaccharide of the starch glycogen class by *Neisseria perflava* as shown by tests upon sucrose and glucose broth cultures\*

BACTERIA	POLYSACCHARIDE FROM SUCROSE	NUMBER OF STRAINS	TESTS (1 TO 6) ON SUCROSE BROTH CULTURES							TESTS (1 TO 5) ON GLUCOSE CULTURES	
			(1) Bacterial sediment darkened by iodine	(2) Opalescence	(3) Precipitate with 12 vols alcohol	(4) Intensity of color with iodine†		(5) Precipitation vs antiserums‡			(6) Number with free reducing sugars
						Green filter	Red filter	Dextran reactive	Levan reactive		
<i>Neisseria perflava</i>		17	+	+++	+++	300-000	82-284	0	0	14	0
		9	+	++	++	115-208	24-80	0	0	6	0
		8	+	+	+	55-99	10-38	0	0	7	0
		4	+	±	±	15-35	3-6	0	0	3	0
Controls											
<i>N. perflava</i> , 19 34	Amylopectin		+	+++	+++	425	142	0	0	+	0
<i>Streptococcus</i> H F90A	Dextran		0	+	+++	0	0	10 000	0	+	0
<i>S. salivarius</i> , S20B	Levan		0	+	0	0	0	0	2 000	+	0

\* Test 1 was made on the bacterial sediment, whereas tests 2 to 6 were made on the supernatant culture fluids.

† The figures represent colorimeter scale readings.

‡ Dilution of culture fluid that gave precipitation. 0 indicates negative reaction with 1:10 dilution of fluid.

§ The supernatant fluids of some of the strains showed a slight (±) opalescence and alcohol precipitate.

|| An abundant precipitate was obtained with 2.5 volumes of alcohol; this did not occur with the glucose culture fluid.

are features also of dextran and levan formation and were shown by the two representative strains of streptococci in table 3. The polysaccharide formation by the *neisseriae*, however, was distinguished from both dextran and levan formation by the iodine and serological tests. Since the antidextran and antilevan serums that were used were capable of detecting even minute amounts of the polysaccharides, the failure of the sucrose culture fluids of the *neisseriae* to react with them is good evidence that no dextran or levan was present as an accompanying substance in the *Neisseria* cultures.<sup>1</sup>

<sup>1</sup> Although the negative tests with these serums constitute valid evidence of the absence of either dextran or levan, they do not furnish any evidence on the serological properties of the amylopectinlike polysaccharide itself. When solutions prepared from sucrose broth

A point not shown in table 3 is that the sucrose broth culture fluids of all the *Neisseria* strains lost their opalescence and their capacity to give color with iodine when treated with diluted saliva. This effect is illustrated in figure 4. The lytic action of saliva ( $\alpha$ -amylase) may be taken as evidence that the sucrose-derived material is in the starch-glycogen class, although it does not exclude the possibility that small amounts of some other polysaccharide may also be present.

#### POLYSACCHARIDE PRODUCTION BY WASHED BACTERIAL CELLS

Washed bacterial cells obtained from glucose broth cultures, and containing little or no preformed polysaccharide, were found to have the capacity of producing the polysaccharide when incubated with solutions of sucrose and were used in the following experiment on substrate specificity and the general mechanism. Four liters of a 4-day-old culture in broth containing 0.2 per cent glucose were centrifuged, the *Neisseria* cells were washed 3 times with sterile distilled

TABLE 4

*Properties of incubated mixtures of Neisseria cells with various substrates*

SUBSTRATE	SEDIMENT		SUPERNATANT FLUID					
	Dark color with I <sub>2</sub>	Polysaccharide	Opalescence	Precipitate with 1.5 vols alcohol	Dark color with I <sub>2</sub>	Polysaccharide	Free reducing sugars	Inorganic P
		mg per ml				mg per ml	mg per ml	mg per ml
Sucrose	++++	1.48	+++	+++	+++	0.61	2.75*	<0.01†
Glucose-1-phosphate	+	0.13	0	±	0	0.08	0.05	0.04
Other sugars‡	0	0.05	0	±	0	<0.06	—	<0.01†

\* Fluid gave positive qualitative Ekkert (1928) test for free fructose

† The total acid-soluble P was less than 0.05 mg per ml

‡ Glucose, glucose + fructose, lactose, maltose, trehalose,  $\alpha$ -methylglucoside, raffinose, melibiose, melezitose

water and finally suspended in 25 ml of sterile distilled water. Equal amounts of this bacterial suspension were incubated at 23 C for 3 days in a mixture with 0.1 M solutions of sucrose, glucose-1-phosphate, and various other sugars in maleate buffer at pH 6.4. The sucrose was a sample of beet sugar known to be free of the amylopectinlike material that is present as an accompanying substance in many lots of reagent and commercial sucrose (unpublished observa-

culture fluids of *neisseriae* (or solutions of the purified polysaccharide) were mixed with the diluted antisera for the purpose of serological examination, rapid destruction of the starchlike material was found to occur even though the sera had been "inactivated" by heating at 56 C for 30 minutes. The destruction, which was evidenced by loss of opalescence and of capacity to darken with iodine, doubtless was due to the presence of amylase, it occurred with the sera of normal as well as immunized animals. Conditions that will prevent the amylolytic activity of serum without impairing its antibody activity will have to be established before serological study can be made of the *Neisseria* polysaccharide. Amylolytic destruction of the test antigens may have been an unrecognized source of error in past work on the serological activity of polysaccharides of the starch-glycogen class.

tions) The glucose-1-phosphate, kindly furnished by Drs Summerson and du Vigneaud of the Department of Biochemistry, was the crystalline dipotassium salt obtained from potatoes, it contained a trace of starch

The incubated mixtures were centrifuged and chemical analyses made on the bacterial sediments and the supernatant fluids. The polysaccharide contents of the sediments were determined by a micro modification of Pfluger's (1905) method for glycogen, in the case of the fluids the reducing sugar contents of the precipitates obtained with 1.5 volumes of alcohol were determined before and after hydrolysis for 2 hours at 100 C by 1.0 N HCl, and the amount of polysaccharide was calculated as 0.9 of the difference. The inorganic and total acid-soluble phosphorus and the free reducing sugar contents of the fluids were determined by the Fiske and Subbarow (1925) and the Hagedorn and Jensen (1923) methods.

The data in table 4 show that the sucrose yielded considerable amounts of iodine-coloring polysaccharide both in the supernatant fluid and in the bacterial sediment, that the glucose-1-phosphate<sup>2</sup> yielded some but much less than the sucrose, and that none of the other sugars yielded any demonstrable amounts. The data obtained in this experiment with the washed bacterial cells furnished better evidence for the substrate specificity of the polysaccharide synthesis than could be obtained by experiments with growing cultures, because the concentration of the bacterial agents and the pH were constant in all of the test mixtures.

In the sucrose test mixture in which abundant polysaccharide formation had occurred there was no accumulation of inorganic phosphorus, and the total phosphorus concentration was low. These points, together with the much greater yield of polysaccharide from sucrose in comparison to that from glucose-1-phosphate, suggest that, when sucrose is the substrate, glucose-1-phosphate is not required as an intermediate substance. Decisive evidence was obtained in other experiments conducted in the presence of high concentrations of inorganic phosphate. In test mixtures in which the molecular concentration of inorganic phosphate was increased 6 or 8 times that of the substrates, the formation of polysaccharide from glucose-1-phosphate was suppressed, whereas the formation from sucrose was not inhibited.

In regard to the general mechanism of the action on sucrose, the points to be emphasized in table 4 are that the reducing sugar liberated into the fluid is fructose, as indicated by the strongly positive Ekkert (1928) test, and that the amount of the reducing sugar corresponded reasonably well with the amount of polysaccharide in the fluid plus that in the sediment. These features suggest that the synthesis of amylopectinlike polysaccharide by the neisseriae is the same general type of reaction as the synthesis of dextran by *Leuconostoc* enzymes (Hehre, 1943, 1946), that is, that  $n$  molecules of sucrose are converted to  $n$  molecules of fructose plus polysaccharide containing  $n$  glucose anhydride units.

<sup>2</sup> The capacity to form starch from glucose-1-phosphate is influenced by the conditions of growth of the cultures from which the resting cells are obtained. When obtained from agar slant cultures or from cultures grown in broth containing higher concentrations of glucose, the bacteria were more reactive with glucose-1-phosphate than were the resting cells used in the experiment presented in table 4.

Further work with cell-free enzyme systems is now in progress to obtain more definite information on the mechanism

#### DISCUSSION

The experiments dealt with the formation of polysaccharide material of the starch-glycogen class by certain neisseriae from human throats. The capacity was possessed both by cultures in nutrient media and by suspensions of washed cells. A prominent feature was the substrate specificity in systems containing sucrose abundant amounts of the polysaccharide were produced, in systems containing glucose-1-phosphate some, but much smaller amounts, were formed, none of the other sugars tested yielded any of the polysaccharide. The pronounced preference for sucrose in comparison to other common sugars distinguishes the polysaccharide formation by these types of *Neisseria*, not only from the production of intracellular granules of iodine-coloring "reserve carbohydrate," which has long been known to occur with various bacteria and fungi, but also from the production of extracellular amylose by *Torulopsis* recently reported by Mager and Aschner (1947).

*Neisseria* strains possessing the capacity of synthesizing starchlike polysaccharide from sucrose are apparently common in human throats. They represent a larger part of the normal flora than would appear on the usual blood agar plate culture because the development of their colonies is inhibited by accompanying streptococci. A selectively inhibitory medium is required to reveal the real frequency of their occurrence, in our experiments sucrose agar containing penicillin was used. The polysaccharide-forming neisseriae that we examined were reasonably similar to one another in respect to taxonomic properties and agreed fairly well with the definition of *Neisseria perflava* in *Bergey's Manual* (1939). It does not follow of course that there is a regular correlation between the polysaccharide-forming property and the taxonomic properties described for the present *Neisseria* species. However, there is a need for new features in the differentiation of the "pharyngeal" group of *Neisseria*, and simple tests for the production of iodine-coloring polysaccharide from sucrose might prove useful in the description of strains.

Since sucrose and polysaccharides of the starch class are abundantly distributed in nature and constitute our principal source of carbohydrate food, any reaction by which one is converted into the other is of general biological interest. In the case of the *Neisseria* reaction, sucrose seems to be converted to amylopectinlike material by a process of polycondensation of the glucose units and liberation of the fructose units. Glucose-1-phosphate is not required as an intermediate substance, contrary to the current belief that all substances belonging to the starch or glycogen class are necessarily formed from that phosphorylated sugar. Nevertheless, the synthesis of amylopectinlike polysaccharide by the neisseriae and the syntheses of starch and glycogen by the plant and animal phosphorylases have the same fundamental point of similarity previously shown between starch synthesis by the phosphorylases and dextran synthesis by *Leuconostoc* enzymes (Hehre, 1943). That is, the substrate (sucrose or glucose-1-phosphate) in each

instance contains the basic unit of the final polymer product in the form of a glucoside radical that is exceedingly easily split off with acids. Our previous studies (1943, 1946) with enzymes from *L. mesenteroides* and with resting cells of *N. perflava*, together with the work of Hestrin and Avineri-Shapiro (1944) with levansucrase from *Aerobacter levanicum*, established the fact that sucrose enters reactions in which its glucoside and fructoside units are transferred, apparently without the mediation of phosphorylated compounds, to polysaccharides of several types (dextran, levan, and amylopectinlike material). More recently it has been shown that the glucoside unit of sucrose can be transferred to disaccharides as well as to polysaccharides (Doudoroff, Barker, and Hassid, 1947). Taken together, all of the foregoing studies, made with bacteria or with bacterial enzymes, show that sucrose is a versatile donor of glycoside groups, and it is of interest to speculate on whether glycoside transfer reactions involving sucrose occur in plant or animal tissues, and especially whether reactions like that described in the present paper may not have importance in plant metabolism.

#### ACKNOWLEDGMENT

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#### SUMMARY

Certain types of *Neisseria* commonly occurring in the normal throat and nasopharynx and belonging to the species *N. perflava* (Bergey *et al.*) have been found to form large quantities of a polysaccharide of the starch-glycogen class when grown with sucrose. None of the polysaccharide was produced when other common sugars were substituted for sucrose, so that the present phenomenon is obviously different from the long-known production of "reserve carbohydrate" by various bacteria and fungi, but closely resembles the traditional "viscous fermentation" of sucrose involved in the formation of polysaccharides of the dextran and levan types. From the results of experiments with washed bacterial cells as well as with actively growing cultures it appears that the synthesis represents a polycondensation reaction of sucrose involving the transfer of glucoside groups to polysaccharide, with the liberation of free fructose, in a way similar to that shown for the synthesis of dextran from sucrose by enzymes from *Leuconostoc mesenteroides*.

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# THE PRODUCTION OF 2,3-BUTYLENE GLYCOL BY *AEROBACTER AEROGENES* 199<sup>1</sup>

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2,3-Butylene glycol is a fermentation product that can be obtained, in good yield, from a large variety of carbohydrate substrates. A study of the 2,3-butylene glycol fermentation was made during the war because the glycol could serve as a starting material for the production of butadiene. This work has been presented in reports exchanged among laboratories engaged in butylene glycol research.

The development of butylene glycol fermentation has been treated by Ledingham, Adams, and Stanier (1945). To bring their review of the literature to date it is necessary to add the work of Perlman (1944) on the production of glycol from wood sugar and the work of Ward, Pettijohn, Lockwood, and Coghill (1945) on the production of glycol from acid-hydrolyzed starch.

The bacterial strain, *Aerobacter aerogenes* 199, used in all of the experiments was obtained from the Northern Regional Research Laboratory at Peoria, Illinois. The products that this organism produces vary widely with the conditions under which it is grown. An anaerobic fermentation of 100 millimoles of glucose by this organism yielded 42.4 mm of 2,3-butylene glycol, 79 mm of ethyl alcohol, 1.5 mm of acetoin, 12.8 mm of acetic acid, 8.5 mm of formic acid, 16.8 mm of lactic acid, 7.4 mm of succinic acid, 139 mm of carbon dioxide, and 41.6 mm of hydrogen (Olson, 1945).

Any variation of the fermentation conditions brings about a change in the amount of the individual products formed. Mickelson and Werkman (1938) using *Aerobacter indologenes* found that pH has a marked effect on the fermentation products. When the pH of the fermentation was maintained above 6.3, the result was an accumulation of acetic and formic acids, a decrease in the amount of carbon dioxide and hydrogen liberated, and a lowering of the yield of 2,3-butylene glycol and acetoin. Kluyver and Scheffer (1933) found that higher concentrations of carbohydrate could be fermented with a decreased fermentation time if air was passed through the medium. Under aerobic conditions there was a marked change in the product distribution from that of the anaerobic fermentation.

Under optimum conditions 80 mm of 2,3-butylene glycol and 1.8 mm of acetoin are formed per 100 mm of glucose fermented. The ratio between the two varies

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with the amount of air passed through the fermentation. The glycol has been found to be converted to acetoin in the presence of the organism when large volumes of air are passed through the fermentation after the sugar has disappeared.

#### METHODS

*Analytical* The sugar determinations were made by the Shaffer-Somogyi (1933) method and were corrected for the presence of acetoin. The starch content of the grain used is expressed as glucose and was determined by both the A O A C direct acid hydrolysis method and the A O A C diastase-acid method.

The acetoin present in the fermentations was determined by a modification of the method of Langlykke and Peterson (1937). It was found that an undiluted sample distilled in the Duclaux apparatus frothed excessively. The amount of acetoin present in the fermentation liquor was so low that to be within the range of the determination it was necessary to use an undiluted sample. Therefore the method was revised to measure smaller amounts. The fermentation liquor could then be diluted before distillation and frothing was eliminated. A sample containing from 0.2 mg to 1.0 mg of acetoin was pipetted from the third quarter of the distillate into a  $\frac{5}{8}$ -by-8-inch test tube. To this were added 2 ml of 1 N sodium hydroxide and 5 ml of 0.02 N iodine solution. After 10 minutes the sample was acidified and titrated with 0.005 N sodium thiosulfate. It was found that 2,3-butylene glycol interfered with the determination, but if the sample was distilled twice, the low distillation constant of the glycol and the high distillation constant of the acetoin increased the ratio of the acetoin to the glycol so much that the interference by the glycol was insignificant.

2,3-Butylene glycol was determined by the periodate oxidation method of Johnson (1944) on the extract after ether extraction at pH 6. It was found that fermented wheat mashes frothed excessively during the ether extraction. To avoid this difficulty the samples were treated as follows: 3 ml of a 20 per cent copper sulfate solution and 2 ml of a 20 per cent calcium hydroxide suspension were added to 10 ml of sample in a test tube. After thorough mixing, the samples were heated for 10 minutes in a boiling water bath and centrifuged. Five ml of the supernatant were pipetted into the sample cup of the ether extraction apparatus and extracted for 18 hours. The glycol was then determined on the extract. The copper sulfate and calcium hydroxide treatment has the advantage that the acetoin is destroyed quantitatively. The method gives an over all recovery of approximately 102 per cent as indicated in table 1. Table 2 shows the destruction of acetoin by the treatment.

This method was used to analyze all wheat mash fermentations until the following method was developed by David Blake at the University of Wisconsin (Olson, 1945). Blake's method may be used for determining both 2,3-butylene glycol and acetoin and is probably somewhat more specific for the glycol than the methods commonly used. The apparatus used is diagrammatically represented in figure 1. Steam entering the jacket forces the 0.5-ml sample in the sample cup up into the sample column, where it is intimately mixed with the steam, which volatilizes the glycol and acetoin. In the wash column, which contains water,

the glycol is removed from the steam, but the acetoin passes through to the column containing acidified potassium periodate. Here the acetoin is oxidized to

TABLE 1  
*Effect of copper sulfate and calcium hydroxide treatment on glycol recovery*

SAMPLE	GLYCOL PRESENT	APPARENT GLYCOL CONTENT AFTER PRECIPITATION AND EXTRACTION	RECOVERY
	g/L	g/L	per cent
Glycol and water mixture	22.80*	23.37	102.0
Glycol and water mixture	22.80*	23.43	102.4
Fermented culture	34.55†	35.60	103.0
Average			102.4

\* Determined before extraction

† Determined after the extraction of a diluted aliquot in a large extractor

TABLE 2  
*Destruction of acetoin by copper sulfate and calcium hydroxide treatment*

ORIGINAL ACETOIN CONCENTRATION	ACETOIN AFTER TREATMENT*	DESTRUCTION
g/L	g/L	per cent
10.52	0.10	99.0
10.52	0.11	99.0
21.06	0.00	100.0
21.06	0.00	100.0
42.12	0.04	99.9
42.12	0.02	99.9

\* Corrected for dilution by reagents

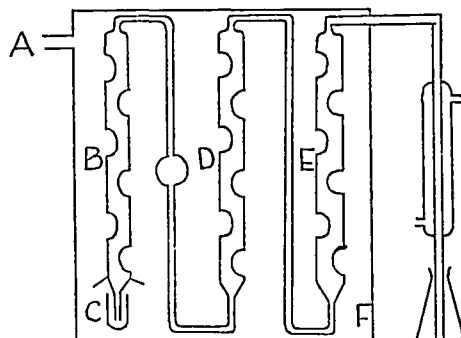


FIG. 1 BLAKE'S APPARATUS FOR DETERMINATION OF 2,3-BUTYLENE GLYCOL AND ACETOIN

A, steam inlet, B, sample tower, C, sample cup, D, wash column, E, periodate column, F, jacket

acetaldehyde, which is distilled into bisulfite solution in the receiver and determined in the usual manner (Johnson, 1944). Acidified potassium periodate solu-

tion is now added to the wash tower, and the distillation continued. The acetaldehyde resulting from the glycol oxidation is caught in the bisulfite solution and titrated.

In actual practice the wash tower consists of seven sections, each consisting of a tube (12 by 150 mm) with hemispherical indentations. Each tube contains about 15 ml of water. Because of the large difference in steam volatility of acetoin and glycol, the acetoin progresses through this fractionating system much more rapidly than does the glycol. The acetaldehyde from the acetoin is recovered in the first 15 ml of distillate, acidified potassium periodate is then added to the wash tower and the acetaldehyde from the glycol is recovered in the next

TABLE 3

*Comparison of Blake's method for butylene glycol determination with Johnson's method*

CULTURE (FERMENTED WHEAT MASH)	BUTYLENE GLYCOL CONTENT		DIFFERENCE
	Blake's method*	Johnson's method†	
I	g/L	g/L	per cent
	38.9	39.6	+1.5
	39.1		
Avg	39.0		
II	37.7	36.3	0
	35.5		
	35.6		
	36.4		
Avg	36.3		
III	37.0	37.9	+0.5
	38.1		
	37.9		
Avg	37.7		

\* Recovery of 98.3 per cent of the glycol in the second fraction was assumed.

† Average of duplicate determinations, corrected for acetoin.

25 ml. The seven-section wash tower is not quite adequate for complete separation of glycol and acetoin, but the distillation is so reproducible that a small correction factor may be safely applied. The accuracy of the method is indicated in table 3.

**Hydrolysis.** The wheat used was a standard grade of Dark Northern Spring no. 2 and was obtained from the Commodity Credit Corporation. The fineness of grind is indicated in table 4. The starch content of the wheat, expressed as glucose, was 73.2 per cent by the A O A C diastase-acid method and 80.1 per cent by the A O A C direct acid hydrolysis method. All values are expressed on a dry grain basis.

The following is an outline of the procedure used for hydrolysis of raw wheat

mashes in the cooker shown in figure 2 One and one-half liters of a 25 per cent wheat mash were placed in a pyrex battery jar, Corning 6940 Hydrochloric

TABLE 4  
*Fineness of grind of the wheat used for hydrolysis*

MESH OF SCREEN	WHEAT PASSING THROUGH SCREEN
	<i>per cent of total</i>
20	57 0
40	18 0
60	9 5
80	11 25
100	4 0

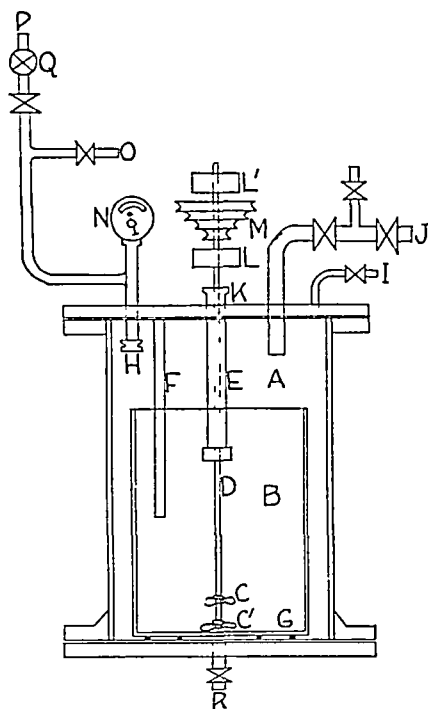


FIG 2 APPARATUS FOR AGITATED ACID HYDROLYSIS OF WHEAT MASHES

A, hydrolysis chamber, B, glass reaction vessel, C, C', stainless steel propellers, D, stainless steel shaft, E, lower bearing with packing gland, F, thermometer well, G, reaction vessel supports to allow passage of steam and air around bottom of reaction vessel, H, steam director to prevent the condensed steam from diluting the mash, I, outlet to permit the displacement of the air by steam at the beginning of hydrolysis, J, outlet to blow down condenser which was not used in this work, K, upper bearing and packing gland of stirrer, L, L', ball bearings of stirrer, M, step pulley to vary speed of stirring, N, pressure gauge, O, air inlet, P, steam inlet, Q, reducing valve used to regulate the steam pressure in the chamber, R, outlet used in cooling

acid was used at a concentration of 0.19 N The pyrex jar containing the mash was then placed in the hydrolyzer After the cover was closed, steam was intro-

duced through steam inlet H. The air present and the condensate formed during the heating time were allowed to escape through valve R. The stirrer, consisting of two three-bladed propellers, one and one-half inches in diameter, revolved at 1,150 rpm. The time required for the mash to reach temperature varied with the pressure of hydrolysis, from 2 minutes at the lower pressures to 3 minutes at the higher pressures. The holding time has been varied at each pressure. At the end of the holding time the steam was turned off, high-pressure air was introduced through H, and at the same time outlet valve R was opened as much as possible without lowering the pressure in the chamber below that used during hydrolysis. The cooling time (to 100 C) varied between 10 and 15 minutes depending upon the pressure used for hydrolysis. This method of cooling made it

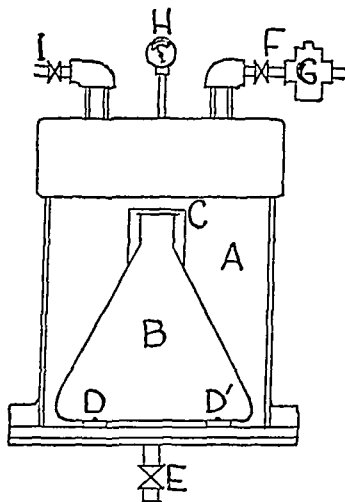


FIG 3 APPARATUS FOR ACID HYDROLYSIS OF WHEAT MASHES WITHOUT AGITATION

A, steam chamber, B, 500-ml flask, C, beaker for prevention of dilution of mash from condensed steam, D, D', supports for flask to permit more even heat penetration, E, condensate outlet valve, F, steam inlet, G, reducing valve, I, air inlet

possible to recover the mash quantitatively, so that the amount of grain per liter of mash was accurately known

*Stationary acid hydrolyzer* The hydrolyses run in the hydrolyzer shown in figure 3 were done by a procedure similar to that used in the hydrolyzer in figure 2 with the following exceptions (1) no stirring was used, and (2) the mash volume used was 200 ml in a 500-ml Erlenmeyer flask with a beaker covering the mouth to prevent the accumulation of condensate in the mash

*Fermentation* All glucose fermentations were run in 5-gallon glass bottles as shown in figure 4. The volume of medium fermented was 12 liters. The medium as devised at the Northern Regional Research Laboratory contained (in g per liter) glucose, 100,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25,  $\text{KH}_2\text{PO}_4$ , 1.8, urea, 2.0,  $\text{CaCO}_3$ , 5.0. The  $\text{KH}_2\text{PO}_4$ , urea and  $\text{CaCO}_3$  were sterilized individually and added at the time of inoculation

Forty ml of sterile "vegifat" Y (antifoam produced by the National Oil Products

Company) were added to the fermentation at the time of inoculation and, in addition, 10-ml quantities were added as required. "Vegifat" Y appeared to be toxic when large amounts were required to keep the froth under control. One fermentation was run with 3 per cent octadecanol in lard oil. This antifoam did not show any toxic effect (table 6). Figure 5 shows the decrease in yield per hour of glycol with increased amounts of "vegifat" Y. The temperature was automatically controlled. The temperature fluctuations could be limited to 0.5 C.

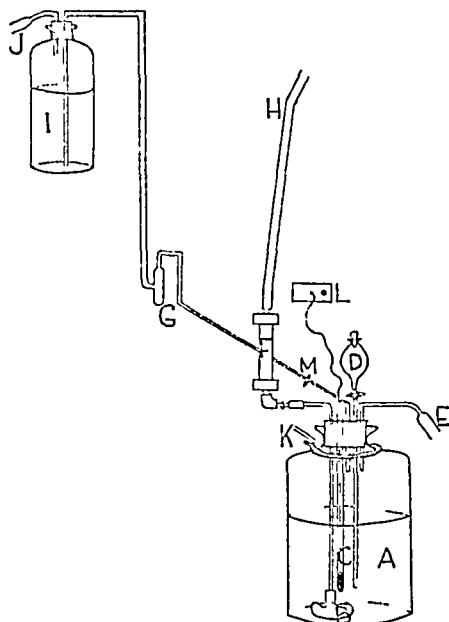


FIG. 4. APPARATUS FOR PRODUCTION OF 2,3-BUTYLENE GLYCOL BY THE SLOW-FEED METHOD.

A, 5 gallon glass carboy, fermentation vessel, B, kidney-shaped canvas aerator, C, temperature control well with mercury thermostat, D, separatory funnel with tube reaching below surface of liquid used for sampling and the addition of antifoam, E, cotton-filtered air outlet, F, cotton filled air sterilizer, G, flowmeter to measure flow of liquid from I, H, inlet for saturated air from flowmeter, I, 9-liter serum bottle with sugar concentrate for slow feed fermentation, J, filtered air inlet, K, perforated copper ring used for cooling, L, primary relay for temperature control, M, cock for regulating the flow of sugar concentrate.

Slow-feed fermentations were run to determine the concentration of glycol that could be reached before the fermentation was inhibited. During the slow-feed fermentations the solution in the 9-liter serum bottle (figure 4, I) contained 450 g per liter of glucose, and potassium phosphate and urea in the same proportion to the glucose present as in the regular fermentation medium. This concentrate was added throughout the fermentation at such a rate that the sugar concentration in the fermenter remained close to 3 per cent.

For the fermentation of the acid-hydrolyzed wheat mashes the cooled mash was adjusted to pH 6 with sodium hydroxide and diluted to 12 to 18 g per 100-ml

concentration One-hundred-ml aliquots of the diluted mash in 500-ml Erlenmeyer flasks were autoclaved at 15 pounds' steam pressure for 30 minutes To each flask, after being cooled to 30 C, were added 0.5 per cent  $\text{CaCO}_3$ , 0.3 per cent urea, and 5 ml of inoculum The inoculum medium used was 12 per cent acid-hydrolyzed wheat mash with 0.5 per cent Difco yeast extract, 0.3 per cent

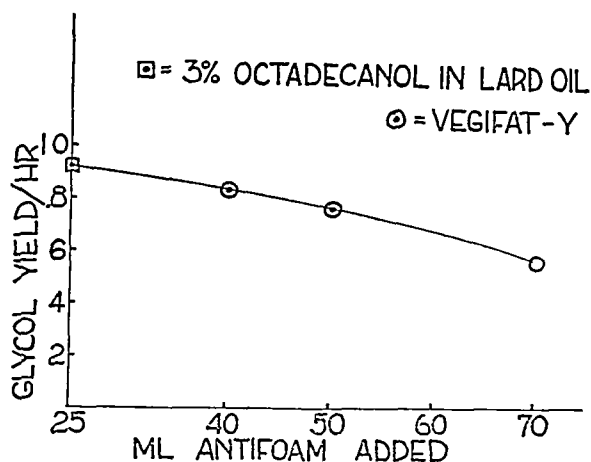


FIG 5 EFFECT OF AMOUNT OF ANTIFOAM ADDED

TABLE 5  
 Comparison of three types of unaerated inocula

INOCULUM DATA		FERMENTATION DATA				
Type of inoculum	Plate count of second stage of inoculum	Sugar used in 1st 12 hours	Sugar used finally	Plate count (24 hours)	Glycol yield on sugar used	Ace.oin yield on sugar used
	million	g/100 ml	per cent	million	per cent	per cent
A	720	2.3	100	1,840	36	0.7
B	950	2.12	100	1,850	36.2	1.8
C	550	0.38	55	740	—	—

A First stage 30-ml volume—beef extract, 3 g, tryptone, 5 g, yeast extract, 10 g, malt sprouts, 10 g, urea, 2 g, calcium carbonate, 12 g, and water to make one liter Second stage 320-ml volume—same as first stage

B First stage 30-ml volume—cerelose, 100 g, corn steep, 5 g, calcium carbonate, 5 g, yeast extract, 10 g, extract of 20 g of malt sprouts, and water to make one liter Second stage 320-ml volume—cerelose, 100 g, corn steep, 5 g, calcium carbonate, 5 g, extract of 10 g of malt sprouts, and water to make one liter

C First stage 30-ml volume—cerelose, 100 g, corn steep, 5 g, calcium carbonate, 5 g, and water to make one liter Second stage 320-ml volume—same as first stage

urea, and 0.5 per cent  $\text{CaCO}_3$  added The inoculum medium was seeded with *Aerobacter aerogenes* 199 from an agar slant and shaken for approximately 18 hours The inoculated flasks were shaken for 30 hours at 30 C The fermentations were stopped at 48 hours by steaming The shaker used moved the flasks horizontally a total stroke distance of 10.5 cm, completing 94 cycles per minute



## EXPERIMENTAL RESULTS

*Five-gallon bottle fermentations* A 3 per cent inoculum was used in all cases. The type of inoculum medium was varied and found to have a marked effect upon the starting time of the fermentation. This was particularly noticeable when the inoculum was not aerated. The compositions of three inoculum media are given in table 5. The rate at which the fermentation started is indicated in table 5, in the column showing the amount of sugar utilized during the first 12 hours. It was found later that an enriched inoculum medium was not needed if the inoculum was aerated during incubation.

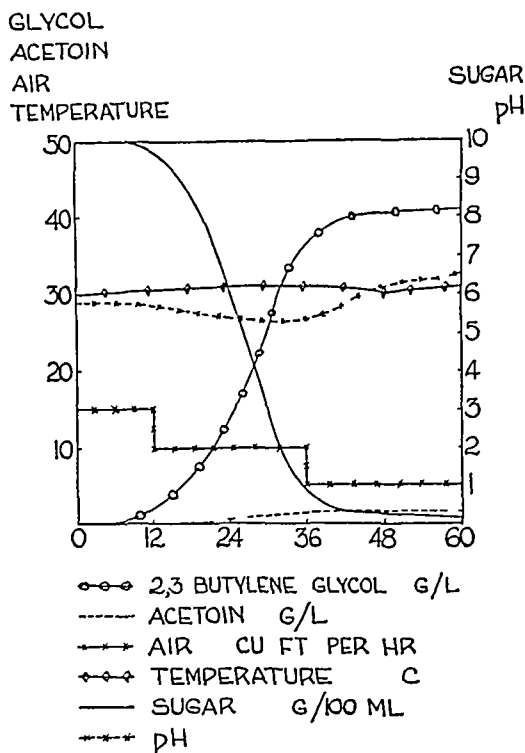


FIG 6 GRAPH OF A TYPICAL GLYCOL FERMENTATION

The course of a typical glycol fermentation is shown in figure 6. Only a small amount of sugar is utilized during the first 12 hours. During the next 24 hours the fermentation proceeds at a rapid rate. The pH drops from 5.9 to 5.25 during the first 36 hours and then rises throughout the remainder of the fermentation.

The temperature at which the fermentation takes place has a marked effect on the glycol production. Thirty degrees Centigrade appeared to be the optimum temperature. This may be an artifact, however, since at the higher temperatures frothing became more persistent and made it necessary to add larger amounts of "vegifat" Y. The decreased yields might have been due to the increased inhibiting effect of the larger amounts of antifoam agent.

It can be seen from table 6 that the aeration rate that gave the best results was 15 cu ft per hour for the first 12 hours, 10 cu ft per hour during the next 24 hours, and 5 cu ft per hour until the end of the fermentation At 30 C this aeration

TABLE 6  
Data obtained from 5-gallon bottle fermentations

RUN NO	INOCULUM	SUGAR USED	GLYCOL BY WT ON SUGAR USED	ACETOIN BY WT ON SUGAR USED	FERMENTATION TIME	VEGIFAT Y ADDED	TEMPERATURE	AERATION	FINAL PH
		per cent	per cent	per cent	hours	ml	degrees C		
1	C	82	31.6	8.84	60	40	30-31	I	6.1
2	C	98	40.0	1.0	48	40	30-31	II	6.1
3	C	98	24.4	7.4	48	40	29.5-36	I	7.37
4	C	97	24.4	7.45	48	45	29.5-36	II	6.37
5	C	99	37.5	0.52	48	50	30	I	5.53
6	C	98.6	36.1	1.08	48	50	30	II	5.00
7	C	63.5	37.2	0	60	70	30	II	
8	C	64.6	25.3	0	60	70	30	II	
9	B	89	38.2	0	60	70	30	II	
10	B	73.2	36.6	0	50	70	29-30	III	5.58
11	B	Frothed out							
12	B	85	33.4	0	50	70	29-30	II	5.34
13	B	91.2	30.9	1.9	50	70	30-35	II	5.21
14	B	87.8	29.3	1.7	50	70	30-35	II	5.30
15	B	86.5	30.6	1.6	50	70	30-35	III	5.25
16	B	82.3	37.6	0	72	70	33.5-35	II	5.60
17	B	97.5	32.4	1.6	42	80	33.5-35	IV	5.32
18	B	88.1	35.12	0	48	70	33.5-35	II	5.4
19	B	100	36.2	1.8	52	50	33.5-35	II	5.75
20	A	100	36.0	0.7	48	50	33.5-35	II	5.75
21	C	90.7	42	0	81	50	30	II	5.83
22	C	98.8	39	0	72	50	30	II	5.33
23	C	98.8	39	0	72	50	30	II	5.60
24	C*	99.8	40.5	0	44	25†	30	II	5.8
25†	D	95	38.2	0.63	33	30	30	II	5.5

Aeration I—10 cu ft per hour throughout fermentation, II—15 cu ft per hour for first 12 hours, 10 cu ft per hour next 24 hours, and then 5 cu ft per hour to the end of fermentation, III—10 cu ft per hour first 36 hours, 5 cu ft per hour to the end, IV—23 cu ft per hour first 30 hours then 5 cu ft per hour to the end of the fermentation

\* Aerated

† Three per cent octadecanol in lard oil was used instead of "vegifat" Y

‡ This was a fermentation of a 12 g per 100 ml acid-hydrolyzed wheat mash. The conditions of hydrolysis were the same as those of runs 25 and 26 in table 9. The inoculum also was the same as that used in all hydrolyzed grain fermentations.

rate, compared with one of 10 cu ft per hour throughout the entire fermentation, gave a higher average yield of glycol and a lower average yield of acetoin, as may be seen from table 6

The slow-feed fermentations described in table 7 were run at 30 C. Runs A2

and A3 showed that by the slow addition of a sugar concentrate over a period of 108 hours, over 265 g of glucose per liter of beer were fermented yielding a solution containing approximately 9.8 per cent glycol. The amount of glucose fermented was approximately the same as that reported by Christensen (1944). At the beginning the 5-gallon bottles contained 4.69 liters of glucose solution at a concentration of 5.925 g per 100 ml. Fermentation was allowed to proceed until the sugar concentration was approximately 3 per cent (at 20 hours) and kept near 3 per cent thereafter by a slow addition of sugar concentrate. The average yield of glycol and acetoin in runs A2 and A3 was 42.1 per cent, based on the weight of the glucose fermented. It may be seen by comparing tables 6 and 7 that the glycol was formed at the same rate in the slow-feed fermentation as in the regular batch fermentation.

TABLE 7  
*Concentrations of glycol attained in slow feed fermentations*

RUN NO	SUGAR FERMENTED	FERMENTATION TIME	CONCENTRATION OF GLYCOL ATTAINED	ACETOIN FORMED	YIELD OF GLYCOL AND ACETOIN ON WT OF SUGAR USED	AERATION
	g/L	hours	g/L	g/L	per cent	
A1	196	72	76.3	3.2	38.0	A
A2	265	108	99.04	9.32	41.9	A
A3	266	108	96.7	14.37	42.3	B
A4	184	84	60.5	0	32.9	C
A5*	194	108	89.2	0	46.0	B

Aeration A—15 cu ft per hour for first 14 hours, 10 cu ft per hour thereafter, B—20 cu ft per hour first 12 hours, 10 cu ft per hour next 4 hours, 7.3 cu ft per hour thereafter, C—15 cu ft per hour for first 12 hours, 5 cu ft per hour next 10 hours, 2 cu ft per hour next 2 hours, no air next 16 hours, 5 cu ft per hour to end of fermentation, D—15 cu ft per hour first 10 hours, approximately 5 cu ft per hour thereafter.

\* The glass tubes used in aeration and sampling were replaced by galvanized iron pipes.

*Hydrolyzed grain fermentations* A number of hydrolyzed grain fermentations in which the hydrolysis was performed in the hydrolyzer of figure 3 are summarized in table 8. In all these hydrolysis experiments a uniform increase was made in the concentration of acid for each increase of grain mash concentration between the acid concentration of 0.132 N for a mash and 13.46 g per 100 ml and 0.264 N for a mash of 35.88 g per 100 ml. Table 8 and figure 7 show the marked decrease in glycol yield with increasing mash concentration. It may be seen from the table that hydrolysis at a high mash concentration gave a good yield only if the fermentation was carried out at a low concentration. Run 9 gave a poor yield, but when the mash was diluted before fermentation (run 10) the yield was equal to that obtained in other runs at the same mash concentration (runs 3 and 4).

The optimum hydrolysis conditions for the hydrolyzer of figure 3 were found to be 50 pounds' pressure for 30 minutes at the acid concentration of 0.132 N with a mash of 13.46 g per 100 ml. A mash hydrolyzed under these conditions when fermented gave an average yield of 24.9 g of glycol per 100 g of grain. With the hydrolyzer in figure 2 a mash better suited for fermentation was ob-

tained The stirring during hydrolysis decreased the required time of hydrolysis to one-third This may be seen by comparing runs 1 and 2, table 8, with runs 17 and 18, table 9 The mash hydrolyzed (unstirred) at 50 pounds for 30 minutes

TABLE 8  
*Effect of mash concentration on fermentability*

RUN NO	HYDROLYSIS		INCUBATION		APPARENT SUGAR		GLYCOL YIELD	FINAL pH
	HCl conc	Mash conc	Total time	Time aerated	Original	Final		
	N	g/100 ml	hr	hr	g/100 ml	g/100 ml	g/100 g dry grain	
1	0 132	13 46	48	30	8 95	0	26 22	5 65
2	0 132	13 46	48	30	9 75	0	23 63	6 18
3	0 149	17 94	48	30	10 89	0 71	21 68	5 73
4	0 158	17 94	48	30	12 50	1 40	22 00	6 28
5	0 167	22 46	48	30	13 03	1 85	19 03	5 84
6	0 185	22 46	48	40	15 13	5 30	15 40	5 85
7	0 185	26 91	48	30	15 08	3 05	12 00	5 85
8	0 211	26 91	54	48	15 41	11 41	7 41	5 60
9	0 264	35 88	54	48	16 91	15 21	1 12	5 00
10*	0 264	35 88	48	40	8 45	1 02	21 96	6 05
		14 94						

Hydrolysis pressure, 50 pounds, hydrolysis time, 30 minutes, mash unstirred

\* Mash was diluted to twice its volume

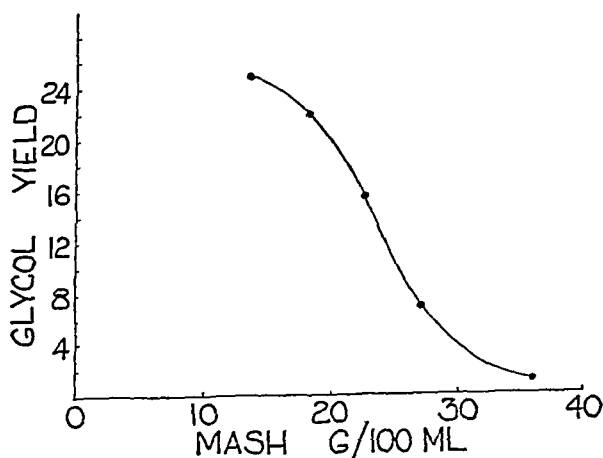


FIG 7 EFFECT OF MASH CONCENTRATION ON GLYCOL YIELD

when fermented gave a glycol yield of 24.9 g per 100 g of grain A mash stirred while being hydrolyzed at 50 pounds for 10 minutes when fermented gave a glycol yield of 24.4 g per 100 g of grain

The data obtained from a series of stirred hydrolyses indicate that at the acid

TABLE 9  
*Effect of hydrolysis conditions on glycol yield*

RUN NO	HYDROLYSIS TIME	STEAM PRESSURE	GLYCOL YIELD PER 100 G DRY GRAIN	
			Flask A	Flask B
	min	lb per sq in	g	g
1	14	25	21 05	21 78
2	18	25	22 69	23 00
3	22	25	18 95	18 24
4	8	30	19 75	19 77
5	10	30	22 80	21 95
6	14	30	22 60	21 90
7	18	30	25 48	24 58
8	22	30	19 90	19 77
9	6	40	20 25	19 80
10	8	40	22 35	23 95
11	10	40	22 80	22 91
12	14	40	24 44	25 43
13	18	40	27 81	28 33
14	22	40	23 80	24 60
15	6	50	20 67	20 79
16	8	50	24 52	24 56
17	10	50	24 99	23 75
18	10	50	24 23	24 50
19	12	50	24 81	24 74
20	14	50	25 42	25 50
21	14	50	25 57	25 40
22	18	50	26 60	28 82
23	6	60	23 52	22 62
24	8	60	23 94	23 92
25	10	60	28 63	28 40
26	10	60	27 00	26 80
27	12	60	29 00	28 50
28	14	60	25 00	25 49
29	6	70	21 40	22 43
30	8	70	27 61	27 20
31	10	70	23 25	23 59
32	14	70	22 57	22 75
33	6	85	20 72	19 70
34	8	85	24 12	24 40
35	10	85	22 93	23 00
36	6	100	19 70	19 85
37	8	100	24 44	24 46

Mash conc , 13.46 g per 100 ml    Acid conc , 0.19 N HCl

concentration of 0.19 N only pressures between 40 and 70 pounds yielded hydrolyzates that could be fermented to give 28 or more g of glycol for each 100 g of grain. Run 27 in table 9 in which a yield of 29 g of glycol per 100 g of grain was obtained is a yield equivalent to the best yield reported by Ward, Pettijohn, Lockwood, and Coghill (1945) on a pure starch hydrolyzate.

#### SUMMARY

*Aerobacter aerogenes* fermentation of glucose to 2,3-butylene glycol with aeration gave yields under optimum conditions of 80 millimoles of glycol per 100 millimoles of glucose. Sugar concentrations up to 10 per cent were fermented in less than 48 hours. Sugar concentrations up to 26.5 per cent (108 hours) were fermented when concentrated substrate was added continuously during the fermentation. Glycol concentrations up to 98 g per liter were produced in the slow-feed fermentations.

Yields of 28 g of glycol per 100 g of grain were obtained when acid-hydrolyzed wheat mashes were fermented.

The stirring of wheat mashes during hydrolysis made it necessary to hydrolyze only one-third as long as with unstirred mashes.

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# THE NUTRITION OF CLOSTRIDIUM KLUYVERI

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When a culture medium containing ethanol, calcium carbonate, and the usual inorganic salts is inoculated with black mud and incubated anaerobically at 30 C, a spontaneous fermentation soon begins that often results in the conversion of much of the alcohol to a mixture of acetic, butyric, and caproic acids (Barker, 1937b). A considerable quantity of methane is also evolved. In such fermentations the dominant organisms are *Methanobacterium omelianskii* and *Clostridium kluyveri*. The former organism causes the oxidation of ethanol to acetic acid and the coupled reduction of carbon dioxide to methane, whereas the latter organism has been shown to be responsible for the formation of butyric and caproic acids (Barker and Taha, 1942). Until recently, however, it was not possible to obtain an adequate understanding of the chemical reactions catalyzed by *C. kluyveri* because pure cultures of this organism could not be grown except in very complex media, and even then growth was scanty and the yield of caproic acid was low. As a preliminary to further metabolic studies it was necessary therefore to investigate the nutrition of the organism in some detail. In the present paper it is shown that *C. kluyveri* has unusual nutritional requirements that can be satisfied by the use of a relatively simple, completely synthetic medium.

## EXPERIMENTAL PROCEDURES AND RESULTS

**Basic growth requirements** In previous studies appreciable growth of *C. kluyveri* could only be obtained in media containing inorganic salts, a reducing agent such as thioglycolate, ethanol, and an abnormally high concentration of yeast autolysate (Barker and Taha, 1942). An earlier attempt to replace the yeast autolysate by some other nutrient was unsuccessful, so the conclusion was reached that yeast autolysate contains one or more substances of special nutritive value for *C. kluyveri* that are not present in most other complex media. At the beginning of the present investigation it was therefore decided to fractionate yeast autolysate in order to identify its active constituents. The following experiments were done with strain K 1.

The first fractionation procedure was a separation of volatile acids by steam distillation. It was at once found that the distillate was highly active in stimulating growth in a basal medium containing ethanol and a small quantity (1 vol per cent) of yeast autolysate, whereas the residue from the steam distillation was completely inactive. By the Duclaux distillation method the volatile acid fraction was shown to consist largely of acetic acid, and the growth-stimulating activity of the fraction could be entirely accounted for by the acetate it contained.

The addition of larger amounts of acetate to the basal medium improved the growth far beyond the highest level previously attained with maximal concen-

trations of yeast autolysate Table 1 shows that growth increases from zero with no added acetate to a maximum when the acetate concentration is about 0.8 per cent

When acetate is added in excess, the amount of growth is dependent upon the supply of ethanol Table 2 illustrates this relation With 1 per cent sodium acetate and 0.8 per cent ethanol, rapid and abundant growth is obtained in an otherwise adequate medium

When adequate amounts of ethanol and acetate are provided only a small quantity of yeast autolysate or other similar material is required For example,

TABLE 1  
*The effect of acetate concentration on growth\**

CH <sub>3</sub> COONa 3H <sub>2</sub> O ADDED %	MAXIMAL TURBIDITY†
0.0	0
0.1	14
0.2	36
0.4	60
0.8	64

\* The medium contained the inorganic salts of medium 1, 0.4 per cent ethanol, and 20 vol per cent of acetate-free yeast autolysate

† Measured with a Klett-Summerson photocolormeter

TABLE 2  
*The effect of ethanol concentration on growth\**

ETHANOL ADDED g/100 ml	MAXIMAL TURBIDITY† (2 - log G) × 1 000
0.0	0
0.1	27
0.2	51
0.4	70
0.8	104

\* Medium 1 contained 0.075 per cent Difco yeast extract, 1.0 per cent sodium acetate, and the indicated amounts of ethanol

† Measured with an Evelyn photocolormeter

50 mg of Difco yeast extract per 100 ml of medium are sufficient to allow maximal growth Even this small amount of yeast extract can be entirely replaced by two growth factors, biotin and *para*-aminobenzoic acid, provided they are added at the rate of 0.3 µg and 5 µg, respectively, per 100 ml

In common with many other microorganisms, *C. kluyveri* requires carbon dioxide as a nutrient In our early experiments, which were carried out entirely in test tubes, no carbonate was added directly to the medium but some carbon dioxide was supplied by the pyrogallol-carbonate seal used to remove oxygen When the organism is grown under such conditions in a liquid medium, it pro-



duces a uniform turbidity that reaches a maximum in 24 to 48 hours. The cells are actively motile and generally occur singly. Only after growth has ceased do they begin to settle and form a conspicuous sediment. Large cultures of 1 to 10 liters in the same medium behave quite differently even when provided with a small pyrogallol-carbonate seal. The bacteria develop much more slowly and occur mainly as large clumps of immotile cells resting on the bottom or adhering to the walls of the vessel. The bulk of the medium remains perfectly clear. Sometimes such cultures even fail to develop at all. These phenomena were found to be due to a deficiency of carbon dioxide. The same type of growth can be obtained in test tube cultures by replacing the carbonate in the anaerobic seal with sodium hydroxide or by using a solution of a chromous salt instead of pyrogallol as an oxygen absorbent.

The addition of a small amount of sodium carbonate to the medium entirely eliminates the above-mentioned signs of carbon dioxide deficiency in both large and small cultures. The quantity of sodium carbonate required for maximal growth is of the order of 0.1 to 1 mg per 100 ml. For routine cultures we have made a practice of adding 10 mg  $\text{Na}_2\text{CO}_3$  per 100 ml medium.

On the basis of the foregoing results the following synthetic medium (medium 1), which supports excellent growth of *C. kluysteri*, was developed: ethanol, 0.8 g, sodium acetate hydrate, 0.8 g, m/1 pH 7.0  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffer, 2.5 ml,  $(\text{NH}_4)_2\text{SO}_4$ , 50 mg,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 20 mg,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 1 mg,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 mg,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.25 mg,  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.25 mg, biotin, 0.3  $\mu\text{g}$ , *para*-aminobenzoic acid, 5  $\mu\text{g}$ , sodium thioglycolate, 50 mg,  $\text{Na}_2\text{CO}_3$ , 10 mg, and glass-distilled water, 100 ml. The thioglycolate can be replaced by 20 mg  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , which is best added after autoclaving. When a strictly synthetic medium is not required, the growth factors may be replaced by 50 to 100 mg Difco yeast extract. It is best to inoculate the medium very soon after it is autoclaved. Since *C. kluysteri* is an obligate anaerobe, the culture medium must be protected from oxygen by means of a pyrogallol-carbonate seal or some similar device. When an active inoculum is used, good growth is obtained in 24 hours at 35 C.

*Utilization of organic substrates other than ethanol and acetate.* After the fact that both ethanol and acetate are needed as macronutrients had been established, the possibility of replacing these substrates by structurally related compounds was explored.

In testing for acetate substitutes a basal medium containing an excess of ethanol (1 per cent) was used and the compound to be tested was added in a concentration of 0.1 to 0.4 per cent. In some experiments, when it was thought that substrates to be tested might be inhibitory in such concentrations, a small, limiting amount of acetate, usually 0.1 per cent, was added both to the experimental culture and to the control. Either growth stimulation or toxicity could then easily be detected. In all experiments growth was measured with either a Klett-Summerson or an Evelyn photocolormeter.

The following compounds were tested: propionate, butyrate, valerate, caproate, lactate, pyruvate, glycine, *alpha*-alanine, *beta*-alanine, and *alpha*-aminobutyrate. Positive results were obtained in these experiments only with propionate and

butyrate, although evidence has been obtained from metabolic experiments (Bornstein and Barker, 1948) that valerate can also be utilized to a slight extent as a substitute for acetate. Typical results obtained with several fatty acids are given in table 3.

In concentrations below 0.5 per cent, propionate is almost as good a substrate as acetate. Growth occurs rapidly and is roughly proportional to the amount of propionate added. At concentrations above 0.5 per cent, propionate is somewhat inhibitory as compared with acetate and both the rate of growth and yield of cells are less. The bacteria can still multiply, however, in media containing as much as 2 per cent sodium propionate and 2 per cent ethanol. Two per cent acetate is also somewhat inhibitory, though reduced growth occurs even with 2.5 per cent acetate and ethanol. With high substrate concentrations a period of

TABLE 3

*The growth of Clostridium kluyveri on ethanol and higher homologues of acetic acid\**

SODIUM SALT ADDED (g/100 ml)		MAXIMAL TURBIDITY†
Acetate	0.2	38
"	0.4	70
Propionate	0.1	16
"	0.4	78
"	0.1, acetate 0.2	53
Butyrate	0.2	20‡
"	0.1, acetate 0.2	42
Valerate	0.1	0
"	0.1, acetate 0.2	32
Caproate	0.1	0
"	0.1, acetate 0.2	32

\* Medium 1 was used containing 0.65 per cent ethanol, 0.5 vol per cent yeast autolysate, plus the indicated fatty acid salts.

† Measured with a Klett-Summerson photocolormeter.

‡ Growth began after 35 days' incubation.

adaptation appears to be required, two or three transfers in the same medium are needed before maximal growth is attained.

Butyrate is not as effective a substrate as acetate or propionate. It is readily metabolized by *C. kluyveri* when a small amount of acetate is also provided, but in the absence of the latter a long lag period is often observed before the bacteria begin to develop normally. The best growth with butyrate is only about half that obtainable with an equimolar quantity of acetate. This is to be expected since the catabolism of acetate involves two steps, acetate to butyrate to caproate, whereas with butyrate as a substrate only the second step occurs (Bornstein and Barker, 1948, Barker, Kamen, and Bornstein, 1945).

In testing for ethanol substitutes essentially the same methods were used as in testing for acetate substitutes, except that the medium contained 1 per cent acetate and, in some experiments, a small amount of ethanol. The compounds tested were propanol, butanol, lactate, and pyruvate. Negative results were ob-

tained with all four substrates. Lactate and pyruvate were tested repeatedly under various conditions so that we feel that the inability of strain K 1 to use these compounds is conclusively established. Unfortunately the experiments with propanol and butanol were not done in the presence of ethanol, so that the negative results obtained thus far are not completely conclusive in view of the possibility that even 0.1 per cent solutions of these compounds may be inhibitory to the organism.

*Utilization of carbon dioxide* After carbon dioxide was shown to be essential for the normal growth of *C. kluyveri*, it seemed worth while to look for an actual utilization of carbon dioxide in growing cultures. The possibility of detecting carbon dioxide utilization by chemical means was enhanced by the observation that no carbon dioxide appeared to be formed in the fermentation process.

A preliminary experiment showed that there is a small disappearance of carbon dioxide of the order of magnitude of 0.1 to 0.2 mm per 100 ml of medium 1 and that it is dependent upon the growth of the organism. There was no loss of carbon dioxide from cultures placed in a refrigerator immediately after being

TABLE 4

*The dependence of carbon dioxide utilization on substrate concentration*

SUBSTRATE CONCENTRATION*			INITIAL CO <sub>2</sub>	FINAL CO <sub>2</sub>	CO <sub>2</sub> UPTAKE
	%		mm/100 ml	mm/100 ml	mm/100 ml
Sodium acetate	0.1		0.28	0.20	0.08
" "	0.2		0.28	0.13	0.15
" "	0.4		0.28	0.08	0.20
Sodium propionate	0.1		0.22	0.17	0.05
" "	0.2		0.22	0.12	0.10
" "	0.4		0.22	0.07	0.15

\* In medium 1 containing 0.4 per cent ethanol and 0.5 vol per cent yeast autolysate

inoculated, and when a culture was incubated for a short period until growth started and was then placed at 5 C, the loss was greatly reduced.

A second experiment was designed to demonstrate a possible correlation between the amount of carbon dioxide utilized and the quantity of cells formed. Medium 1 was used, containing 0.01 per cent Na<sub>2</sub>CO<sub>3</sub> and varying concentrations of acetate or propionate. The tubes used as culture vessels were fitted with "oxsorbent" seals and were tightly closed with rubber stoppers during the incubation period. After growth had ceased, the cultures were analyzed for carbon dioxide. The initial carbon dioxide content of the media was obtained by analyzing a second set of inoculated tubes which had been kept at 5 C.

The results of this experiment are presented in table 4. It can be seen that in all cultures a small but significant amount of carbon dioxide disappeared. With either acetate or propionate the carbon dioxide uptake increased with substrate concentration and, consequently, with the yield of cells. By comparison of the data in tables 1 and 4 it may be concluded that there is an almost direct proportionality between cell yield and carbon dioxide uptake with acetate as a sub-

strate By carrying out an ethanol-acetate fermentation in the presence of carbon dioxide labeled with  $C^{14}$ , it was found that at least 70 per cent of the carbon dioxide utilized could be recovered in the washed bacterial cells Little or none of the labeled carbon from the carbon dioxide went into the fatty acids that are the main products of the fermentation (Bornstein and Barker, 1948)

*Enrichment cultures* *C. kluyveri* was originally isolated from black mud by the use of an enrichment medium containing ethanol and calcium carbonate as the main ingredients This medium consistently allows the enrichment of methane-producing bacteria but does not always yield good cultures of *C. kluyveri*

Now that the unique nutritional requirements of *C. kluyveri* are known, its enrichment from natural sources can be achieved with greater certainty and rapidity by the use of medium 1, slightly modified Biotin and *para*-aminobenzoic acid are used in preference to yeast extract since even small amounts of the latter stimulate the growth of a variety of amino-acid-fermenting bacteria that interfere with the isolation of *C. kluyveri* In order to eliminate sulfate-reducing and methane-producing bacteria as far as possible, it is desirable to replace most of the sulfate in medium 1 by chloride and to reduce the carbonate to 1 mg per 100 ml In this way a highly specific enrichment medium is obtained The inoculum should be pasteurized to eliminate nonsporulating bacteria and, of course, anaerobic conditions must be maintained

By the use of this enrichment method we have obtained active cultures of *C. kluyveri* in a few days from black mud Such cultures contain relatively few bacteria of other types The isolation of pure cultures from the enrichments is very easily accomplished by the shake culture technique

#### DISCUSSION

The most notable result of the present investigation is the demonstration that an anaerobe belonging to the group of butyric acid bacteria can satisfy its energy requirements by metabolizing a mixture of ethanol and acetate All other butyric acid bacteria require a more complex substrate such as a carbohydrate, lactate, or pyruvate (Bhat and Barker, 1947), or one of the amino acids (Barker, 1937a) Not only does *C. kluyveri* not require such compounds but it is unable to utilize them In order to grow anaerobically it must have ethanol and acetate or one of its close homologues These substrates are more or less quantitatively converted into a mixture of higher fatty acids (Barker, 1947, Barker, Kamen, and Bornstein, 1945, Bornstein and Barker, 1948)

So far no substrate has been found that can be substituted for ethanol Even propyl alcohol does not appear to be utilized by *C. kluyveri*, though there is still a slight possibility that it can be metabolized under special conditions Lactate and pyruvate are definitely excluded as ethanol substitutes

Acetate can be replaced by only two compounds tested thus far, propionate and butyrate Fatty acids with longer chains do not support growth, though there is evidence that valerate may be metabolized to a limited extent According to present information any alteration of the acetic acid molecule, other than the substitution of a methyl or ethyl group for one of the hydrogens, destroys its usefulness for this organism

There is nothing unusual about the fact that *C. kluyveri* requires carbon dioxide for growth, but its ability to cause a net disappearance of carbon dioxide is noteworthy. Only a relatively few heterotrophic bacteria can do this, since such organisms generally form more carbon dioxide than they consume. Most of the carbon dioxide fixed by heterotrophic anaerobes studied thus far is found in catabolic products. *C. kluyveri* is exceptional in this respect since most of the assimilated carbon is present in the bacterial cells.

The demonstration that *C. kluyveri* needs two simple organic compounds for its energy metabolism raises the question regarding the existence of other obligate anaerobes having similar nutritional requirements. A few such organisms are already known. Perhaps the first to be reported was *Clostridium sporogenes*, which grows on a mixture of amino acids, some of which are oxidized while others are reduced (Fildes and Richardson, 1935, Stickland, 1934). A similar type of energy metabolism has been established for *Clostridium botulinum* (Fildes, 1935, Clifton, 1940). Recently the growth of *Clostridium lacto-acetophilum* on lactate and glycerol was shown to be dependent upon the presence and simultaneous utilization of acetate (Bhat and Barker, 1947). The substrate requirements of this organism are very similar to those of *C. kluyveri*, differing only in the substitution of lactate for ethanol. Undoubtedly other anaerobic bacteria requiring more than one organic compound for their energy metabolism will be found in the future.

Certainly the best way to find such organisms is by the use of the enrichment culture method. It has been shown that this method can be applied very successfully in the isolation of *C. kluyveri* and *C. lacto acetophilum* from natural sources. Since a two-substrate requirement results from the need for one compound as a reductant and another as an oxidant, enrichment media for new bacteria of this type should contain a pair of potentially oxidizable and reducible compounds as the main ingredients. As reductants, compounds such as simple primary and secondary alcohols, glycols, polyalcohols, fatty acids, amino acids, hydroxy acids, and hydrogen gas might be used. As oxidants, a variety of fatty acids, amino acids, unsaturated and keto acids, ketones, aldehydes, and even polyalcohols could be tested. The number of combinations of oxidants and reductants is almost unlimited. Sugars or other readily fermentable compounds should be avoided since they favor the development of the usual one-substrate organisms.

A study of two-substrate organisms may contribute a great deal to our understanding of anaerobic transformations of organic materials under natural conditions. Such processes nearly always involve a multiplicity of organisms and substrates. Winogradsky (1932) has pointed out certain limitations of the pure culture method when applied to complex problems of soil microbiology. For a complete understanding of microbial processes in nature the interaction of substrates as well as of organisms must be taken into consideration.

#### SUMMARY

It has been shown that *Clostridium kluyveri* grows very well in a synthetic medium containing inorganic salts, ethanol, acetate, biotin, and *para*-amino-

benzoic acid as the main ingredients. No compound has been found that can be substituted for ethanol. Acetate can be replaced by propionate and, less adequately, by butyrate. *C. kluyveri* does not attack glucose, pyruvate, or other common fermentation substrates. Carbon dioxide is consumed during growth, most of it being used for the synthesis of cell constituents. By the use of the synthetic medium the isolation of *C. kluyveri* from natural sources is greatly facilitated.

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# THE IDENTIFICATION OF STREPTOMYCIN ON PAPER STRIP CHROMATOGRAMS

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During the course of an investigation into the antibiotic activities of a considerable number of strains of *Streptomyces* it became apparent that a simple and rapid test for streptomycin would save much time and effort. The paper strip chromatogram method of Consden, Gordon, and Martin (1944) seemed to offer interesting possibilities. This method was originally developed for the identification of amino acids but has been extended by Partridge (1946) and Flood, Hirst, and Jones (1947) to sugars, by Lugg and Overell (1947) to organic acids, and by Nord and Vitucci (1947) to creatine. It has been used also by Goodall and Levi (1946) and by Winsten and Spark (1947) for the identification of analogues of penicillin.

## METHODS

Pilot experiments using commercial streptomycin and also the culture broth from one of the proved strains of *Streptomyces griseus* soon demonstrated that the solvent systems used by the authors cited were not sufficiently selective for streptomycin. It was eventually found that a 3 per cent solution of  $\text{NH}_4\text{Cl}$  would move the streptomycin in a sharp band near the advancing solvent front. The mechanism of this action was investigated by excising sections of the strip, leaching them in water, and determining the relative concentrations of salt by Nesslerization using a Klett-Summerson photoelectric colorimeter to determine the ammonia content. The streptomycin is deposited at a critical salt dilution which is in the neighborhood of 0.5 per cent. This was proved by running strips in a series of dilutions until the streptomycin band became diffuse.

Neither ninhydrin nor Somogyi's reagent (1945) were satisfactory to develop a colored band on the paper strip. Silver nitrate was, of course, useless with culture filtrates. It was found, however, that a slight modification of the Sakaguchi reaction (1925) gave excellent results. The strip is removed from the chamber and dried on a stainless steel screen over a hot plate. It is then sprayed with  $\text{N}/2$   $\text{NaOH}$  and immediately with 0.25 per cent  $\alpha$ -naphthol. After standing 2 minutes it is sprayed with  $\text{NaOCl}$  prepared according to the original directions of Sakaguchi (1925). A brilliant red band is formed with streptomycin. The band is usually sharply outlined with a center at an  $R_f$  value of 0.80 to 0.86 depending upon temperature. The  $R_f$  value is the ratio of movement of the band to the total movement of the solvent. This can be confirmed by excising sections from a parallel strip and testing them on plates of *Bacillus subtilis* or other susceptible organisms. Figure 1 shows the  $\text{NH}_4\text{Cl}$  concentration and appearance of the strip.

During the pilot runs it was found that the apparatus and manipulation could be considerably simplified by running the strip against gravity rather than from a suspended solvent container as described by Consden *et al.* (1944). The simplified apparatus is shown in figure 2. The strips used were no. 2 Whatman paper cut to 12 mm in width. The average length of run from the point where the substance to be distributed was applied was 170 mm. A pencil mark was drawn 65 mm from the bottom, and one drop of the solution to be investigated was applied at that

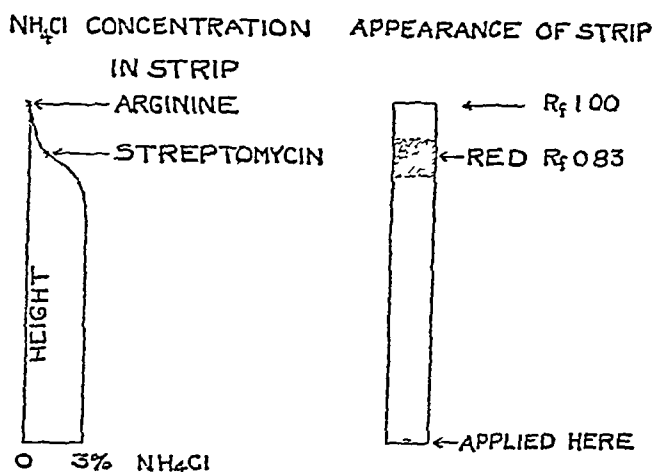


FIG. 1. Left: LEVELS TO WHICH ARGININE AND STREPTOMYCIN ARE CARRIED BY SOLVENT IN PAPER STRIP. Right: STREPTOMYCIN ZONE IN DEVELOPED PAPER STRIP.

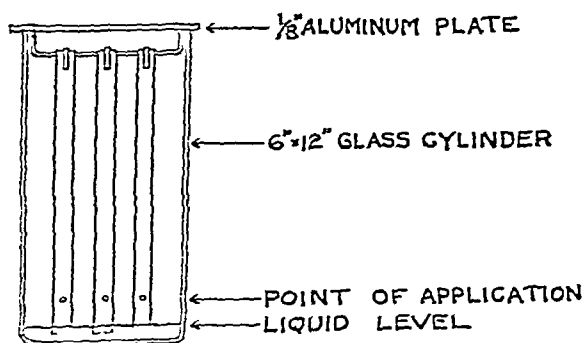


FIG. 2. APPARATUS FOR PAPER STRIP CHROMATOGRAMS AGAINST GRAVITY.

point. The spot was then dried on a hot plate to prevent seepage back into the solvent solution. After drying, the strip was hung in the chamber with the end submerged in the solvent solution, and the chamber was sealed with vaseline to maintain a saturated atmosphere. The time to complete a run varied with the solvent used and the temperature, but was usually from 4 to 12 hours. Solutions containing 30  $\mu\text{g}$  per ml of streptomycin gave excellent reactions.

When the solvent front had reached the desired height, the strip was dried to drive off water and solvent and to fix the constituents in the strip. Unknown



antibiotics were run with both  $\text{NH}_4\text{Cl}$  solutions as here described and usually with butyl alcohol and water mixtures as described by Consden *et al* (1944)

Experiments on unknown broth cultures of various organisms were made on parallel strips with 3 per cent  $\text{NH}_4\text{Cl}$ . One strip was developed with Sakaguchi's reagent and the other cut into eight sections and tested on *Escherichia coli* test plates. In some cases three strips were run and tested on both *E. coli* and *B. subtilis* plates. At the same time two or three strips were run with butyl alcohol saturated with water. These were developed with ninhydrin and colored bands correlated with the activity shown on test plates.

#### EXPERIMENTAL RESULTS

1 Pilot runs on streptomycin using 2  $\mu\text{g}$  of streptomycin calcium chloride complex.

a Pure water showed no distribution. The streptomycin diffused in a wide band in both directions from the point of application.

b Butyl alcohol and water and phenol water systems showed no distribution.

c Pyridine and  $\text{NH}_4\text{Cl}$  showed a sharp band at an  $R_f$  value 0.83.

d Three per cent  $\text{NH}_4\text{Cl}$  alone gave substantially as good results as it did with the addition of pyridine.

e Other salts such as  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaCl}$  gave somewhat more diffuse bands but at the same  $R_f$  value.

2 A broth filtrate from a strain of *S. griseus* furnished through the courtesy of Merck and Company of Rahway, New Jersey, gave a band identical to that obtained with commercial streptomycin.

3 A run was made with arginine to eliminate the possibility of confusing free arginine with streptomycin. This gave an extremely sharp band at  $R_f$  1.00.

4 Culture filtrates from *Streptomyces* 17C, isolated in this laboratory, showed good activity against *E. coli* and *B. subtilis*. No streptomycin could be demonstrated when run with  $\text{NH}_4\text{Cl}$ . A run with butyl alcohol and water developed with ninhydrin showed a pink band at  $R_f$  0.2 that was confirmed on *B. subtilis*.

5 Culture filtrates from *Streptomyces* M17-N3, isolated in this laboratory, showed high activity against *B. subtilis* but none against *E. coli*. A run with  $\text{NH}_4\text{Cl}$  showed a diffuse pink band at  $R_f$  0 that was confirmed on *B. subtilis*. A run with butyl alcohol and water gave a diffuse yellow band at  $R_f$  1.00 that was confirmed on *B. subtilis*.

6 Culture filtrates from *Streptomyces* 2B, isolated in this laboratory, showed fair activity against *E. coli* and *B. subtilis*. A run with  $\text{NH}_4\text{Cl}$  gave the typical streptomycin reaction, which was confirmed on *E. coli* test plates. A run with butyl alcohol and water showed a pink band at  $R_f$  0, which was also confirmed on *E. coli* test plates.

#### DISCUSSION AND CONCLUSIONS

The chromatographic method here described is apparently highly selective for streptomycin. The mechanism involved is obviously a salting-out process, but more detailed information concerning it is still under investigation. In the

meantime it is being used to advantage to detect streptomycinlike substances in culture filtrates. In connection with the other procedures described in the literature cited it is also used to indicate successful methods of extraction, since the behavior of unknown substances on paper strips with various solvents classifies them in this respect.

*Streptomyces* 2B, mentioned above, produces very small quantities of antibiotic in shake cultures and somewhat more in stationary broth cultures. On agar plates, however, it shows unusual activity with the typical streptomycin bacteriostatic spectrum. Work on it would have been abandoned had the chromatographic method not given evidence that it produces a streptomycinlike substance.

It would seem possible that the paper strip method of studying salting-out effects at low salt concentrations might be useful in biochemical fields other than antibiotics.

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## SCREENING METHODS FOR DETERMINING ANTIBIOTIC ACTIVITY OF HIGHER PLANTS<sup>1</sup>

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Plants and their extracts have been used for centuries as remedies for the cure or alleviation of disease. Bigelow (1818) in his monographs depicts the toxicities and simple chemical reactions of numerous plant extracts. More recent investigators (Boas, 1934, Boas and Steude, 1935, Keding, 1939, Lucas and Lewis, 1944, Osborn, 1943, Schmidt, 1942, Seegal and Holden, 1945, Sanders *et al*, 1945, Irving *et al*, 1946, Little and Grubaugh, 1946, Heatley, 1944, Southam, 1946) have found many plants to contain substances active *in vitro* against both gram-negative and gram-positive bacteria and fungi. Carlson, Bissell, and Mueller (1946) have shown several of these plant extracts to be active *in vivo* against malarial and bacterial infections. The authorities used for classification of the plants collected were Peck (1941) and Britton and Brown (1913).

The available literature indicates that the majority of investigators have tested plant juices or water extracts on seeded agar plates in which zones of inhibition and diffusion were observed. It is the purpose of this report to relate other types of solvents which may be used to recover potential antibiotic substances from plants.

*Methods of extraction.* Fresh green plants were collected and stored in the cold room until prepared for testing. Those portions of the plant to be tested were macerated in a mortar or Waring blender after the addition of a volume of 0.9 per cent sodium chloride solution, equivalent to one-half the amount of plant material. Separate extractions were made of the flowers, stem, leaves, root, and seeds or buds of larger plant specimens. After the macerated plant suspension had been allowed to stand at room temperature for a period up to 1 hour, portions of it were placed in four large test tubes. To the first tube was added an equal amount by volume of 1.5 per cent sulfuric acid, to the second tube an equal volume of solution buffered at pH 4.0, to the third tube an equal volume of solution buffered at pH 9.0, and to the fourth tube an equal volume of ethyl ether. The contents of each tube were thoroughly mixed and placed in the cold room along with the saline extract for 24 hours. Just prior to being tested for antibiotic activity, the 1.5 per cent sulfuric acid extract was neutralized with 4 per cent sodium hydroxide, the supernatant being used for assay.

Removal of dissolved chlorophyll in the ether extract was accomplished by adsorption on charcoal (norite A) or kaolin. Two per cent by volume of the adsorbing agent was thoroughly mixed with the ether extract and allowed to

<sup>1</sup>This investigation has been aided in part by a grant from the United States Navy, Office of Naval Research.

stand at room temperature for 1 hour. Filtration was employed for the removal of the adsorbing agent.

**Method of assay.** The Oxford cup method of assay for antibiotic substances was employed throughout these studies. Freshly isolated strains of *Escherichia coli* and *Staphylococcus aureus* (hemolytic) were used as the test organisms. One ml of an 18-hour broth culture was added to 250 ml of the medium, and 20 ml of this shake culture were poured into sterile petri dishes. The lids of the petri dishes were left ajar for 30 minutes to allow drying of the agar surface. Sterile porcelain cylinders were placed on the surface of the seeded agar and filled with the aqueous plant extracts. Six drops of the ether extract, both before and after the removal of chlorophyll, were placed directly on the seeded agar and allowed to evaporate. The charcoal and kaolin used as adsorbing agents were tested in the same manner. The plates were incubated at 37 C for 24 hours.

With all aqueous solutions, zones of inhibition were measured in mm. In testing ether extracts and adsorbing agents, zones were noted as to completeness of inhibition and degree of diffusion of antibiotics. Stimulation of growth of the test organism was also recorded.

## RESULTS

Table 1 summarizes the results obtained using extracts of 14 plants against *Escherichia coli* and *Staphylococcus aureus*. Saline extracts of 6 of the 14 plants contained substances which were bacteriostatic or bactericidal in character. One plant of this group, *Allium cernuum*, contained antibacterial substances markedly effective against both the gram-negative and gram-positive organisms used. The other five saline extracts were observed to have only fair activity against either the gram-negative or the gram-positive organisms.

The acid-soluble fractions from seven plants (1.5 per cent sulfuric acid, neutralized) were observed to be active inhibitors of bacteria. The extract of *Humulus lupulus* was found to inhibit completely the test organisms with good diffusion of the active substances. The inhibitory activity of the six remaining acid extracts was only partial with little or no diffusion.

Eight of the pH 4.0 extracts of the plants were found to be active against the test organisms. *Rudbeckia laciniata* and *Allium cernuum*, pH 4.0 buffered extracts, inhibited both test organisms with good diffusion of the active agent.

Using a buffered solution of pH 9.0 as an extractive, it was observed that eight of the plants inhibited the growth of the test organisms in varying degrees. The extract of *Rudbeckia laciniata* inhibited both organisms with 3 plus activity. The extracts of *Physalis subglabrata* and *Humulus lupulus* showed greater inhibitory activity against the gram-positive organism than against the gram-negative organism.

The ether extracts of ten of the plants were observed to contain active substances, as indicated by the inhibition of the test organisms. Five of these extracts were inhibitory to both test organisms, with the remaining five having substances active against only the gram-positive organism. It was noted that this

solvent revealed the presence of active substances in *Hypericum multilum*, leaf, stem, and root of *Abutelon abutelon*, and stem of *Silphium perfoliatum*, whereas the aqueous solutions were negative

TABLE 1  
Activity of plant extracts

PLANT	PH OF SA LINE	EXTRACTS INHIBITION									
		Saline		1.5% H <sub>2</sub> SO <sub>4</sub>		4.0 Buffer		9.0 Buffer		Ether	
		Staph	Coli	Staph	Coli	Staph	Coli	Staph	Coli	Staph	Coli
<i>Cannabinaceae</i>											
<i>Humulus lupulus</i>	6.1	-	-	++++	++++	+	++	+++	++P	C-E	C-E
<i>Compositae</i>											
<i>Chrysopsis mariana</i>	6.0	++P	++	+P	+P	++P	+P	++	+	P-E	C-E
<i>Helianthus giganteus</i>	6.4	-	-	-	+	-	-	-	-	-	-
<i>Rudbeckia laciniata</i>											
Stem	5.7	-	+P	-	-	+++	+++	+++	+++	-	-
Leaves	5.3	-	+P	+P	-	-	-	-	-	-	-
Flower	5.2	-	-	+P	+P	-	-	-	-	-	-
<i>Silphium perfoliatum</i>											
Stem	6.3	-	-	-	-	-	-	-	-	P-A	-
Leaves	6.3	++	-	-	-	-	-	-	-	-	-
Flower	4.4	+	-	-	-	-	-	-	-	-	-
<i>Xanthium amers- canum</i>	6.7	-	-	+	+	-	-	++	-	C-G	-
<i>Cornaceae</i>											
<i>Cornus nutallii</i> Aud											
Stem	5.6	-	-	-	-	+	+	-	-	-	-
Leaves	6.2	-	-	-	-	-	-	++	++	-	-
Seed	5.2	-S	-S	-	-	-	-	-	-	-	-
<i>Fabaceae</i>											
<i>Mubomia canadense</i>	6.1	-	+	-	-	+	-	+P	++	P-A	P-A
<i>Hypericaceae</i>											
<i>Hypericum multilum</i>	6.6	-	-	-	-	-	-	-	-	C-E	-
<i>Malvaceae</i>											
<i>Malva rotundifolia</i>											
Stem	6.0	-	-	-	+	++	-	+	-	P-A	-
Leaves	6.3	-	++	-	-	-	-	-	-	P-A	-
Root	5.7	-	-	-	-	-	-	-	-	P-A	-
<i>Liliaceae</i>											
<i>Allium cernuum</i>	5.8	++++	+++	-	-	+++	+++	-	-	C-F	P-F
<i>Smilacina racemosa</i>	5.1	-	-	-	-	-	-	-	-	-	-
<i>Solanaceae</i>											
<i>Physalis subglabrata</i>	-	-	-	+	+	-	-	++++P	+P	C-A	C-A

Legend - no inhibition ++++ 26 mm or better zone of inhibition +++, 19-25 mm zone of inhibition ++, 14-18 mm zone of inhibition +, 9-13 mm zone of inhibition P partial inhibition E excellent diffusion G good diffusion F fair diffusion S, growth stimulated C complete inhibition A, inhibition in area exposed

To rule out the activity of chlorophyll, which might be the inhibitory substance in the ether extracts, several were adsorbed on charcoal or kaolin. The resultant clear ether extracts were tested and the results are depicted in table 2.

Eight plants among those investigated were observed to contain inhibitory

substances in the original ether extracts After adsorption of chlorophyll on charcoal, *Hypericum perforatum*, *Tovara virginiana*, and *Helianthus annuus* ether extracts were found to retain their active inhibitory substances against the test organisms The charcoal did not contain any of the active substances when tested on a seeded agar plate *Ribes bracteosum* Dougl, *Madia elegans* Dougl, and *Ceanothus velutinus* (var *laevigatus* Dougl T G) ether extracts were also observed to retain the active substances after adsorption with charcoal and kaolin When the chlorophyll was adsorbed by charcoal and kaolin, the active agents in the ether extracts of *Bidens frondosa* L (leaf) and *Xanthium pennsylvanicum* (leaf and stem) were removed from solution Further tests confirmed the presence of the active agents in the charcoal and kaolin

#### DISCUSSION

In the search for antibiotic material from higher plants, the possible active substances cannot be foretold, thus a screening technique devised to test various types of extractives would seem indicated An essential step in extractions should be the breaking up of plant cells This may be accomplished through alternate freezing and thawing, enzymatic hydrolysis (autolysis), or maceration (grinding with sand, Waring blender, ball mills, hydraulic press, etc) After cell rupture, several general methods of extraction have been used The following have been tried in this laboratory

(1) Extract with water (or 0.9 per cent saline) This will remove inorganic compounds and a few enzymes It will also remove most albumin, histones, protamines, proteases, peptones, and similar amino acids

(2) Dilute weak acids (acetic, etc) This will remove glutelins, several enzymes, and possibly metaproteins and albuminoids

(3) Dilute weak base (sodium bicarbonate, etc) This will remove principally acidic compounds and glucosides

(4) Weak solutions of strong acids (1.5 to 5 per cent sulfuric acid). This will remove all alkaloids and similar substances of a basic nature

(5) Ether extraction This will remove chlorophyll, waxes, and sterols It will also denature proteins and enzymes

The foregoing five solvents have been used by the authors to test over 300 plants involving more than 1,500 extracts The solvents were selected to yield extracts of varied types of material in which potential inhibitory substances might exist The results of the use of multiple extractions are shown in table 1 If only saline had been used, one plant of the 14 used would have yielded a substance that merited further study, though several of the other aqueous extracts did show slight to fair inhibition By using the other solvents in conjunction with saline, evidence of the presence of some type of inhibitory substance was observed in all plants except one

The use of ethyl ether as a solvent brings up the question whether the dissolved chlorophyll acts as the inhibitory agent This has not proved to be the case, as many plants tested have shown no activity from the ether solutions containing chlorophyll Further evidence to substantiate this observation is shown

in table 2 Several ether extracts of plants were adsorbed on charcoal and kaolin Chlorophyll was removed by the adsorbing agents, and simultaneously in several instances the active principles were partially or completely adsorbed These adsorption experiments appear to indicate the presence of two or more active substances, though at this writing the authors are not able to furnish further information

Sanders, Weatherwax, and McClung (1945) found the juice of *Xanthium pennsylvanicum* to show only fair activity against the gram-negative test organism When using the five suggested solvents, extracts of *Xanthium pennsylvanicum* were found to be inhibitory against both gram-positive and gram-

TABLE 2  
*Activity of ether extracts and adsorption agents*

PLANT	INHIBITION							
	Ether Extracts				Charcoal		Kaolin	
	Before*		After*					
	Staph	Coli	Staph	Coli	Staph	Coli	Staph	Coli
Compositae								
<i>Bidens frondosa</i> L leaf	C-G	C-F	N	N	N	P-A	P-A	P-A
<i>Helianthus annuus</i>	C-F	C-F	C-F	C-F	N	N		
<i>Madia elegans</i> D Don	C-A	C-A	C-G	C-G	P-A	P-A	P-A	C-A
<i>Xanthium pennsylvanicum</i>	P-A	C-P-A	N	N	P-A	P-A	C-P-A	P-A
Hypericaceae								
<i>Hypericum perforatum</i>	C-G	C-E	C-E	C-E	N	N		
Polygonaceae								
<i>Tovara virginiana</i>	C-E	C-G	C-E	C-E	N	N		
Rhamnaceae								
<i>Ceanothus velutinus</i> (var <i>laevigatus</i> Dougl )	C-A	C-A	C-G	C-G	P-A	P-A	P-A	P-A

Legend C, complete inhibition, P, partial inhibition, A, area exposed, F, fair diffusion, G, good diffusion, E, excellent diffusion, N, no inhibition

\* Adsorption

negative organisms The active substances were soluble in saline, ether, and alkaline (pH 9.0) solutions The use of other solvents brought out further evidence of the antibiotic substances present in this plant which were probably not active or in a diffusible state in the pressed juice

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## SUMMARY

The screening of plants for the presence of antibiotic substances would seem to require the testing of extractives prepared by the use of more than one solvent. Extractions with saline, strong acid, ether, weak acid (buffered at pH 4.0), and weak alkali (pH 9.0) were found to be necessary before a plant could be discarded as having no antibiotic activity. The results of the use of various solvents in the extractions of a small series of plants are presented.

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# ANTIBACTERIAL SUBSTANCES SEPARATED FROM PLANTS<sup>1</sup>

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Interest is arising in the possible use of higher plants as the source of naturally occurring antibiotic substances. The use of higher plants in the treatment of disease is as old as the art of healing itself. Isaiah of Biblical times suggested that a plaster of figs be placed on boils as a treatment of this form of infection. Hyssop and cedarwood were used in the cleansing of lepers. The Orientals (Hume, 1940) have used plants and herbs for centuries in the treatment of many diseases.

Preliminary surveys (Osborn, 1943, Lucas and Lewis, 1944, Huddleson *et al*, 1944) of large numbers of plants suggest that many contain potential antibiotic materials. Further work by several investigators (Irving, Fontaine, and Doohttle, 1946, Cavallito, Bailey, and Kirchner, 1945, Heatley, 1944, Cavallito, Buck, and Suter, 1945, Seegal and Holden, 1945) on individual plants or parts of plants indicated that these substances can be purified and remain active in this form against microorganisms *in vitro*. Recently (Carlson, Bissell, and Mueller, 1946) it was demonstrated that partially purified extracts of plants are active *in vivo* against the malaria parasites and against gram-positive bacteria.

During the spring and summer of 1946 it was possible to collect 550 plants in regions of Ohio and Oregon. From these plants, 2,115 extracts were prepared and tested. The object of the study here reported was to determine which plants of those collected from these regions contain active substances which would inhibit the growth of microorganisms *in vitro*.

## EXPERIMENTAL RESULTS

The authorities used for the classification of the plants collected were Peck (1941) and Britton and Brown (1913).

*Preparation of extracts* All plants were used in the fresh state. Those that were shipped long distances were prepared in a manner to minimize excessive drying. On arrival at the laboratory the plants were immediately placed in the cold room. Details of the procedure for the preparation of extracts of the plants, in part or whole, are described in a previous report (Carlson and Douglas, 1947).

*Procedure* Twenty ml of nutrient agar, seeded with an 18-hour culture (1 ml per 100 ml agar) of the test organisms (*Staphylococcus aureus* and *Escherichia coli*), were placed in sterile petri dishes. These poured plates were allowed to dry for 30 minutes. Sterile porcelain cylinders (8 mm by 10 mm high) were placed on the inoculated agar, and prepared extracts of the plants were pipetted into the cylinders. The plates were incubated at 37 C for 24 hours, or for a

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TABLE 1

Activity of plant extracts against *Staphylococcus aureus* and *Escherichia coli*

PLANTS	PH	SALINE		A E		E E		40		90	
		C	S	C	S	C	S	C	S	C	S
<b>Aizoaceae</b>											
<i>Mollugo verticillata</i>	5 47	+									
<b>Alismaceae</b>											
<i>Alisma subcordatum</i>		+					PA				
<i>Sagittaria latifolia</i>											
Leaf	5 4						PA				
Stem	5 7						PA				
<b>Alsiniaceae</b>											
<i>Alsine longifolia</i>	5 6						PA				
<b>Ambrosiaceae</b>											
<i>Ambrosia trifida</i>											
Leaf	6 5	P+					PA				
Stem	6 8	P2+					PA				
<b>Ammiaceae</b>											
<i>Angelica arguta</i>											
Seed	5 17					CA					
Stem	5 8					CA					
Root	5 5					PA	CA				
<i>Deringa canadensis</i>	5 6				+					2+	3+
<i>Sanicula crassicaulis</i>											
Flower	6 0		3+								
Stem and leaf	6 0		2+								
<i>Sanicula greparia</i>		SP2+	2+								
<i>Torilis anthriscus</i>	6 8					CPA	CPA				
<b>Anacardiaceae</b>											
<i>Rhus</i> sp											
Leaf	4 0	P2+	2+								
Flower	4 5	P+	3+	P4+							
<i>Schmalzia crenata</i>	4 1							+		2+	+
<b>Apocynaceae</b>											
<i>Apocynum androsaemifolium</i>	5 78						PA				
<i>Apocynum cannabinum</i>											
Leaf	5 6	3+									
<b>Araceae</b>											
<i>Spathyema foetida</i>											
Leaf	4 2					PA	PA				
<b>Asclepiadaceae</b>											
<i>Acerates viridiflora</i>	6 4					PA	PA				
<i>Asclepias incarnata</i>											
Flower	4 7	+					PA				
Leaf	6 72	+				PF	SCA				
Stem	5 5	+					PA				
<i>Asclepias mexicana</i>											
Stem and root	5 88		+								
Leaf	6 0	+				PA	PF				
<i>Asclepias verticillata</i>	5 6										
<b>Balsaminaceae</b>											
<i>Impatiens biflora</i>	5 7	P+			PA	CA	PCA				
<b>Cannabinaceae</b>											
<i>Humulus lupulus</i>	6 1			3+	4+	CE	CE	2+	+	P2+	3+

TABLE 1 (Continued)

PLANTS	pH	SALINE		A E		E E		40		90	
		C	S	C	S	C	S	C	S	C	S
Caprifoliaceae											
<i>Sambucus canadensis</i>											
Leaf	5 6	+					PA				
<i>Sambucus glauca</i>					+						
Leaf	5 7							+	+	+	
Stem and flower	5 41					PA	PA				
<i>Triosteum perfoliatum</i>											
Stem and root		P2+	P2+								
Leaf		P+	SP3+								
Caryophyllaceae											
<i>Saponaria officinalis</i>	5 5			+	P+						
Chenopodiaceae											
<i>Chenopodium album</i>											
Leaf	6 69	S	P2+								
<i>Salsola pestifer</i>	5 8					PA	PA				
Cichoriaceae											
<i>Lactuca floridana</i>											
Stem and seed	5 7				+						
<i>Nabalus altissimus</i>											
Seed	5 4					PA	PA	P2+	+		
Leaf	5 6					CA	CA	P2+	2+		
Stem	5 6					PA	PA	P+			
Compositae											
<i>Anaphalis margaritacea</i>											
Flower	5 1					PA	PA				
Leaf and stem						CPA	CA				
Root	5 12					PA	CA				
<i>Antennaria plantaginifolia</i>	5 4					PA					
<i>Anthemis cotula</i>							PA				
<i>Arctium minus</i>											
Burrs	5 1						PA				
<i>Aster novae angliae</i>	6 2						PE				
<i>Bidens frondosa</i>											
Leaf stem	5 15	P+	2+			CF	CG		+		
Root	6 0	+	+			PA	PA				
<i>Centaurea jacea</i>	5 1	2+									
<i>Chrysopsis mariana</i>	6 0	2+	P2+	+	P+	CE	PCE	P+	P2+	+	2+
<i>Eupatorium altissimum</i>	6 0										
Leaf and stem	6 3					PF	PF				
Flower	6 4				+	CF	CF				
<i>Eupatorium perfoliatum</i>	6 7						CPE				
<i>Eupatorium purpureum</i>											
Root	5 8	+	+								
<i>Eupatorium urticaefolium</i>	6 0			2+	+	PA	PA				
<i>Grindelia nana</i>											
Flower	5 73					CPF	CPF				
Leaf and stem	6 1					CPF	CG				
<i>Grindelia squarrosa</i>	5 25					PA	PA				
<i>Helianthus annuus</i>											
Stem and root	5 95						PA				
Flower	5 4						CF				
Leaf	7 56						PG				



TABLE 1 (Continued)

PLANTS	PH	SALINE		A E		E E		4.0		9.0	
		C	S	C	S	C	S	C	S	C	S
Cruciferae											
<i>Diplotaxis muralis</i>	5.4						PA				
<i>Lepidium campestre</i>	5.0	P3+									
Leaf and flower	5.5	P3+									
Stem and root											
Euphorbiaceae											
<i>Tithymalopsis corollata</i>	5.16	2+	2+				PA				
Stem and leaf											
Fabaceae											
<i>Meibomia rigida</i>	6.33	2+	+	2+	2+						
<i>Meibomia canadensis</i>	6.1	+				PA	PA	P+	P+		2+
Hydrangeaceae											
<i>Hydrangea</i> sp.	5.11						PA				
Flower											
Hypericaceae											
<i>Hypericum mutilum</i>	6.6						CE				
<i>Hypericum perforatum</i>	5.28						CG				
Root	4.35					PS1	CG				
Leaf, stem, and flower											
Labiatae											
<i>Agastache nepetoides</i>	6.0										
Seed	6.4					PA					
Leaf	6.1					CA	CA		P2+*		
<i>Isanthus brochatus</i>											
Nelumbonaceae											
<i>Nelumbo nelumbo</i>	5.2	+	+								
Leaf	5.52	+									
Stem	5.45	P2+*									
Flower											
Onagraceae											
<i>Gaura biennis</i>	5.8		+				CA				
Stem	5.45						CA				
Leaf	5.8								+		
<i>Oenothera biennis</i>											
Papaveraceae											
<i>Sanguinaria canadensis</i>	5.0			2+	2+	CS1	CS1	2+	CP3+		
Penthoraceae											
<i>Penthorum sedoides</i>	5.8					PA	PA				
Polemoniaceae											
<i>Navarretia squarrosa</i>	5.6					CA	PCA				
Polygonaceae											
<i>Persicaria opelousana</i>	5.72	+				PA	PCE		P+		
<i>Persicaria hydropiper</i>	5.5					PCA	PCA	3+	3+		
<i>Polygonatum corniculatum</i>											
Berry	4.9			2+	2+	PA	PA				
Leaf	5.38						PA				
<i>Ternstroemia scandens</i>	4.7					PA	PA				
<i>Tovara virginiana</i>	4.45						CE				

TABLE 1 (Continued)

PLANTS	PH	SALINE		A E		E E		40		90	
		C	S	C	S	C	S	C	S	C	S
Polypodiaceae											
<i>Polypodium vulgare</i>											
Root	5 35	S	S								
Ranunculaceae											
<i>Paeonia</i> sp											
Flower	5 0	SP3+	SP4+								
Leaf	4 5	P2+	P4+								
<i>Ranunculus recurvatus</i>	5 0	P4+	P3+								
<i>Ranunculus acris</i>	6 0	SP2+	SP2+								
<i>Ranunculus septentrionalis</i>											
Stem and root	6 0	3+									
Leaf and flower	6 0	2+									
Rhamnaceae											
<i>Ceanothus velutinus</i>											
Leaf	5 88	S	S			CA	CA	2+	+		
Stem	5 9							+			
Ribesaceae											
<i>Ribes bracteosum</i>											
Leaf	6 4					PA	PA				
Stem	6 5		+			PA	PA				
Rosaceae											
<i>Agrimonia striata</i>	5 3			2+	+	CE	CE				
<i>Prunus emarginata</i>											
Leaf and berry	6 5					PA	PA				
<i>Rosa</i> sp											
Flower	5 5	SP3+	P4+								
Stem and leaf	6 0	S	P3+								
<i>Spiraea tomentosa</i>	5 6			+		CPG					
<i>Spiraea latifolia</i>	4 65	S	2+	+		PA	SCG				
Rubiaceae											
<i>Mitchella repens</i>	5 0 <sup>+</sup>			+	+			2+	2+		
Scrophulariaceae											
<i>Linaria linaria</i>	5 2					CA	CFA				
<i>Mimulus ringens</i>	5 7						PA		+		
<i>Scrophularia marylandica</i>	5 7	+				PS1	CA				
<i>Verbascum blattaria</i>											
Stem and seed	5 5		2+	2+	+		PA		P2+		
Leaf	6 4						PA				
Root	6 65	+	+	P+					P2+		
Smilacaceae											
<i>Smilax rotundifolia</i>	5 6					PA	PA				
Solonaceae											
<i>Datura stramonium</i>											
Leaf stem and seed	5 6					PA	PA				
<i>Physalis heterophylla</i>											
Leaf	5 1		+				CG		P2+		
Berry	5 0						CA				
Stem	4 8						PF				
<i>Physalis subglabrata</i>				+	+	CA	CA	P+	P+		
Typhaceae											
<i>Typha latifolia</i>	6 3						PG				

TABLE 1 (Concluded)

PLANTS	PH	SALINE		A E		E E		40		90	
		C	S	C	S	C	S	C	S	C	S
Verbenaceae											
<i>Lippia lanceolata</i>	6.8					CG	CG				
<i>Verbena angustifolia</i>	6.3			P+	+	PA	PA				
<i>Verbena hastata</i>											
Leaf and stem	5.35					PA	PA				
<i>Verbena urticifolia</i>											
Leaf	6.4						PE				
Vitaceae											
<i>Vitis bicolor</i>	3.65					PA	PA				
<i>Vitis vulpina</i>											
Seed	3.1	S	S			CA	CA	+			
Leaf and stem	3.92			+	+			+			

Legend C, *Escherichia coli*; S, *Staphylococcus aureus*; AE, acid extract; EE, ether extract; 40, buffer extract; 90, buffer extract; S, stimulation of growth; +, 9-13 mm zone of inhibition; ++, 14-18 mm zone of inhibition; +++, 19-25 mm zone of inhibition; ++++, 26 mm or better zone of inhibition; P, partial inhibition; E, excellent diffusion; G, good diffusion; F, fair diffusion; C, complete inhibition; A, inhibition in area exposed; S1, slight diffusion.

\*Surface inhibition only.

sufficient period to permit accurate measurement of the inhibition or stimulation zones produced. Specific areas were measured and are recorded in table 1 (see legend).

The ether extract (6 to 10 drops) was placed directly on the inoculated agar without the use of the porcelain cylinder. The ether was allowed to evaporate before the petri lid was replaced. The zones of inhibition or stimulation were noted as to completeness and the degree of diffusion of the active agent (see legend of table 1).

**Results.** The effectiveness of the plant extracts in inhibiting *Staphylococcus aureus* and *Escherichia coli* is shown in table 1. In further explanation of the symbols used in the table the following examples are given. The plus signs are self-explanatory in the legend, if they are preceded by "C" the inhibition was complete, and by "P" the inhibition was partial. Under the ether extracts, a plant having the symbol of CPA would indicate that the inhibition was complete to partial in the area over which the ether extract evaporated. The last symbol pertains to the diffusion area, with the preceding symbols indicating the completeness of inhibition.

**Comment.** With ethyl ether as a solvent, many of the plants were found to contain some type of antibiotic substance. At first this was puzzling to the authors, since on several occasions all the plants in a collection would have inhibitory substances in this extract. Adsorption experiments (Carlson and Douglas, 1947) with charcoal and kaolin have shown that chlorophyll was not the active agent. The use of the other solvents brought out the presence of many other active agents against the test organisms that were diffusible in varying degrees on the seeded agar plate.

Another interesting phenomenon observed with several plant extracts was inhibition only on the surface of the agar. Where this was observed, the extract

had not spread over the agar surface. A possible explanation for surface antibiotic interference in these specific cases was oxidation of the diffused agent. The oxidized agent was active while that portion in the agar, not having access to air, remained inactive. This phenomenon was observed with extracts of the flowers of *Nelumbo nelumbo*, extracts of the plants *Schmalzia crenata*, *Isanthus brochatus*, and *Ipomoea pandurata*. Further investigations are being undertaken to study this type of activity in greater detail.

#### ACKNOWLEDGMENT

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#### SUMMARY

The results are reported of a preliminary survey of 550 plants from which 2,115 extracts were prepared. One or more antibacterial substances were separated from 115 of the plants tested. The saline, acid, ether, weak acid (pH 4.0 buffered), and weak alkali (pH 9.0 buffered) extracts of these plants were tested for their antibiotic effectiveness against *Staphylococcus aureus* and *Escherichia coli*.

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# BACITRACIN METHODS OF PRODUCTION, CONCENTRATION, AND PARTIAL PURIFICATION, WITH A SUMMARY OF THE CHEMICAL PROPERTIES OF CRUDE BACITRACIN

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We have previously reported that the antibiotic bacitracin is produced by a gram-positive sporulating bacillus of the *Bacillus subtilis* group isolated from cultures of contaminated tissue removed at operation from a compound fracture of the tibia (Johnson *et al* , 1945) This paper will summarize the results of our work on the production, concentration, and partial purification of bacitracin on a laboratory scale In the experiments dealing with purification processes we have worked with preparations of crude bacitracin supplied to us by several commercial laboratories as well as with material produced and concentrated in this laboratory Studies on the chemical properties of the crude antibiotic were directed toward solving the problem of purification To date no pure bacitracin is available, and the chemical properties of the pure product may prove to differ from those of the crude material we have worked with

## ASSAY OF BACITRACIN

In order to discuss the production of bacitracin, it is necessary to define a measure of potency One unit of bacitracin has been arbitrarily set as the amount which, when diluted 1 in 1,024 under the assay conditions stated below, completely inhibits the growth of our stock strain (Chanin) of group A hemolytic streptococcus

*Serial dilution assay* A beef heart infusion neopeptone broth buffered with sodium dibasic phosphate or a beef heart infusion 2 per cent proteose peptone broth may be used The assay is run in triplicate using 1 in 3, 1 in 4, and 1 in 5 dilutions of the sample in the first tubes of each of three rows of dilution tubes Twofold serial dilutions are made of each of these three solutions far enough along each row to cover the expected limits of potency The tubes are then seeded with 0.1 ml of a  $10^{-2}$  dilution of an overnight culture of the hemolytic streptococcus in blood broth The series is incubated at 37 C, and readings are made after 24, 48, and 72 hours of incubation The end point is the highest dilution in which growth is completely inhibited Thus an end point of 1 in 512 would mean that the sample assayed contained 0.5 units per ml and an end point of 1 in 40, 0.04 units per ml If a closer approximation of the titer than is possible with this dilution scheme is desirable, a series of additional dilutions in the vicinity of the known end point may be set up

Recently a plate method of assay using strains of *Corynebacterium* has been developed independently in this laboratory and in the Cutter Laboratories Details of this method will be published shortly

## PRODUCTION OF BACITRACIN

The factors influencing the production of bacitracin are the composition of the medium, seed cultures, and the conditions of incubation

*Media* We have been chiefly concerned with the production of bacitracin in shallow layers of medium since we have not been successful in obtaining appreciable titers when the growth was submerged with the apparatus at our disposal. In our preliminary work the 1 per cent tryptone broth recommended by Dubos for the production of gramicidin was employed (Dubos, 1939). Production in beef infusion, amigen, and savita broths was also studied. In an attempt to improve the titer of the harvest we also employed the synthetic medium devised by Stokes and Woodward (1943) for the submerged growth of *Bacillus brevis* and Brewer's (1943) soybean digest medium. Table 1 gives the titer obtained after 72 hours' incubation of the seeded media at 37 C. In all experiments the respective media were dispensed in 200-ml amounts in Blake bottles of 1-liter capacity. The bottles were incubated in the horizontal position.

TABLE 1  
Effect of composition of medium on production of bacitracin

MEDIUM	AVERAGE TITER	OCCASIONAL TITER
	units per ml	units per ml
Tryptone	2	4
Beef infusion	2	4
Amigen	2	4-5
Savita	2	4
Synthetic	6	12-16
Soybean digest	12	20-28

The addition of 1 per cent glucose or corn steep liquor to the tryptone or amigen medium did not result in titers comparable to those obtained in the synthetic or soybean digest media, nor did the increase of the amigen content from 1 to 2 per cent raise the titer significantly. Although the soybean digest medium gave the highest titers, this medium was not chosen for concentration and purification studies since it was found that the impurities in concentrates of such harvests were many and were more variable from lot to lot in comparison with concentrates from the synthetic medium.

The formula of the synthetic medium is as follows (final concentration in 1 liter of distilled water)

l-Glutamic acid	5.0 g
KH <sub>2</sub> PO <sub>4</sub>	0.5 g
K <sub>2</sub> HPO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.2 g
MnSO <sub>4</sub> · 4H <sub>2</sub> O	0.01 g
NaCl	0.01 g
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.01 g
CuSO <sub>4</sub> · 7H <sub>2</sub> O	0.01 g
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub>	2 ml sat sol in distilled H <sub>2</sub> O

The medium is adjusted to pH 6.8 to 7 with sodium hydroxide and sterilized after distribution into Blake bottles by autoclaving for 20 minutes at 15 pounds' pressure. After sterilization a concentrated glucose solution previously sterilized by filtration through a Chamberland or Seitz filter is added to the individual bottles to give a final concentration of 1 per cent in the medium. Autoclaving of the medium containing glucose resulted in varying degrees of caramelization of the glucose. In our experience excessive caramelization in such media resulted in poor production of the antibiotic.

When *D*-glutamic acid was substituted for *L*-glutamic acid, one lot of *D*-glutamic acid gave satisfactory results, but the next lot gave negligible titers. The use of *L*-glutamic acid has given consistent yields with no variation in the harvests from lot to lot that could be traced to the acid. We also tried substituting asparagine and glycine for *L*-glutamic acid. Growth in the asparagine medium was poor and the titers were low (0.06 units per ml). Growth in the glycine medium was slow and erratic, although after prolonged incubation (7 to 10 days) the final titers in a few instances compared favorably with those in the *L*-glutamic acid medium after 72 hours' incubation. Sucrose, lactose, and fructose could be substituted for the glucose but gave no consistent or striking rise in titer.

*Seed cultures* Three types of inoculum have been found satisfactory: 3-day surface growth on tryptone agar, 3-day growth in 1 per cent tryptone broth, and spore suspensions. Cultures must be maintained in the rough or mucoid phase since smooth variants produce only small amounts of the antibiotic. Dissociation may be minimized by growing stock or seed cultures on or in media to which no glucose has been added, i.e., tryptone broth or agar.

*Conditions of incubation* The antibiotic is produced at room temperature as well as at 37 C, but a longer period of incubation is required for comparable titers in the harvests. Maximum titers at 37 C are obtained after 3 to 5 days' incubation, but incubation for longer than 96 hours is attended by the risk of autolysis of the pellicle. This autolysis is accompanied by a drop in the titer, which may fall as much as 50 per cent within 24 hours. When cultures were incubated at 35 C, there was no increase in the maximum titer in comparison with control cultures incubated at 37 C. At 35 C the maximum titer was usually attained after 120 hours of incubation.

#### HARVESTING

The pH of the harvest at its maximum titer varies with the composition of the medium. When tryptone broth or soybean digest medium is used, a pH of 7.8 to 8 may be coincident with the maximum titer. When the *L*-glutamic acid medium is used, the pH of the medium drops to 5 to 5.2 during the first 24 to 48 hours of incubation and then rises. The pH of the medium is usually 7 to 7.2 when the maximum titer is reached. If after 72 hours of incubation the pH of the culture is below 6.8, incubation should be continued for another 24 hours.

The antibiotic is harvested by decanting the culture fluid. The pellicle is discarded. The harvest is then clarified by filtration through "standard super-cel." If sterile material is required for assays, the clarified harvests may be filtered through Berkefeld, Chamberland, Selas, or Seitz filters.

## CONCENTRATION

It proved impossible to extract bacitracin from the harvest with the usual organic solvents, such as ether, chloroform, acetone, or ethyl acetate. It was found, however, that 85 to 90 per cent of the activity in the harvest was removed by *n*-butanol. The clarified harvest is extracted twice, using one-half volume of *n*-butanol for each of two extractions. The butanol is then decanted, filtered, and distilled *in vacuo* in the presence of water until all butanol is removed and the volume of the residual aqueous phase is reduced to about 1/100th of the original volume of the harvest. In conjunction with Dr R. E. Steiger we have found that if the temperature of distillation is 28 to 30 C, it is necessary to add 500 ml of water for each liter of butanol extract. Care must be taken that the extract does not become anhydrous at any step of the concentration, as the antibiotic is rapidly inactivated in anhydrous butanol.

## PURIFICATION

In the laboratory small-scale preparation of bacitracin, the following method of purification was found very satisfactory. An equal volume of butanol-ether solution (50 per cent butanol and 50 per cent peroxide-free ether) is added to the aqueous concentrate. The mixture is shaken in a separatory funnel. Concentrated hydrochloric acid is added drop by drop until the mixture separates into layers immediately after shaking. The upper layer becomes darker as the acid is added owing to the removal of impurities from the aqueous layer. The pH of the aqueous layer should be 3 to 4. The lower (aqueous) layer, which contains the active material, is decanted and the extraction is repeated. The mixture is allowed to stand for 10 minutes, and the pH is again tested (it should be 3 to 4). The lower layer is decanted and extracted 5 times with peroxide-free ether, one-half volume of the original concentrate being used for each extraction. The final aqueous layer is distilled under reduced pressure until all butanol and ether have been removed. After distillation the solution is brought to pH 6 to 7 with sodium bicarbonate. The neutralized solution may then be lyophilized. In our laboratory batches the lyophilized material was a yellowish powder with an activity of 10 to 20 units per milligram.

A method of purification recently developed as a result of attempts to purify commercial preparations makes use of the fact that magnesium oxide added to a cold solution of bacitracin precipitates pigmented impurities and carbohydrates without affecting the titer of the bacitracin in solution.

One hundred milligrams of magnesium oxide are added to 10 ml of a faintly acid or neutral solution of concentrated bacitracin. The mixture is stirred and after several hours' storage in the refrigerator is filtered cold. The filtrate is alkaline (pH approximately 8.5). The filtrate is neutralized with hydrochloric acid and the magnesium oxide treatment repeated. The solution again becomes alkaline. If the second magnesium oxide residue is pigmented, the process is repeated again. The antibiotic activity of the magnesium oxide residue is negligible. The original activity is present in the filtrates. Some loss of activity is encountered if the alkaline solutions are permitted to stand at room temperature for 12 hours or more. This alkaline inactivation does not occur if mag-

nesium acetate is used as the precipitant of the pigment, but relatively large amounts of acetate are required, the precipitation is slower, and filtration is more difficult

The bacitracin may then be precipitated as a salicylate by adding salicylic acid to solutions of the antibiotic. No inactivation has been observed. Crude material with an initial potency of 4 to 6 units per mg has yielded salicylates with a potency of 30 to 40 units per mg. These products contained about 12 per cent of combined salicylic acid.

The solution of crude bacitracin is brought to pH 4 with hydrochloric acid, and solid salicylic acid is added, in small portions, until no further precipitation occurs. This first precipitate contains a considerable amount of inactive material, but usually less than 25 per cent of the total antibiotic activity, and it may be discarded. The filtrate is neutralized with sodium bicarbonate. Sodium salicylate or free salicylic acid is then added to complete the precipitation. The precipitate is collected, dried, and repeatedly washed with peroxide-free ether in order to remove any excess of salicylic acid. It is then ground in the funnel and washed again with peroxide-free ether. After drying *in vacuo* at room temperature over phosphoric anhydride, it is weighed and the drying and weighing repeated. When the funnel and its contents show no significant change in weight, the removal of excess salicylic acid is complete. The salicylate is dissolved by percolating 0.05 normal hydrochloric acid through it with rubbing. A small quantity of insoluble impurity remains on the funnel. This acid solution of the salicylate may be assayed directly after suitable dilutions in water are made.

#### CHEMICAL PROPERTIES OF CRUDE BACITRACIN

**Stability** Partially purified neutral or slightly acid (pH 6.6) aqueous concentrates of bacitracin prepared in this laboratory by the butanol method have shown no detectable change in titer after storage for 8 months to 1 year at temperatures of 0 to 5 C. At room temperature there was a loss of 30 to 50 per cent of the activity after a 2 weeks' storage period, but neutral, inorganic-salt-free solutions have been dried at room temperature without loss of activity. Inactivation was complete after 2 weeks at 35 to 37 C. Bacitracin solutions were stable to normal hydrochloric acid at 0 to 5 C but not at 37 C, and to 0.01 normal hydrochloric acid at both 0 to 5 C and 37 C. They were rapidly inactivated in alkaline solution above pH 9 at both temperatures.

The stability of crude bacitracin prepared by several commercial laboratories has varied greatly. Solutions in distilled water, physiological saline, or buffer solutions of some lots of lyophilized material have lost the greater part of their activity when stored for a few days at 0 to 5 C. Solutions of other lots have retained their original activity over a test period of 1 month or longer.

In the presence of hydrogen peroxide there is complete loss of antibiotic activity. The activity shows no measurable change after treatment with the thiol compounds such as hydrogen sulfide or thioglycolic acid. A small but definite reactivation of the peroxide-inactivated bacitracin solutions has resulted from additions to such solutions of thioglycolic acid. Seudi *et al* (1947) have found that bacitracin is partially inactivated by BAL and Na-thiosulfate.

*Solubility* Bacitracin is soluble in methanol, ethanol, isopropanol, *n*-butanol, and cyclohexanol, slightly soluble in cyclohexanone, and insoluble in other organic solvents such as ether, chloroform, benzene, acetone, and ethyl acetate. In aqueous solution the activity is diffusible through a nitrocellulose membrane which holds back particles of molecular weight 2,000 (Seibert, 1928). Some commercial preparations of lyophilized bacitracin have given crystal-clear solutions in distilled water in concentrations as high as 30,000 units per ml.

*Precipitability* (1) *Metal ions* Bacitracin is precipitated by salts of heavy metals. This precipitation is accompanied by inactivation if the heavy metal ions, low in the electromotive series, are used. On the other hand, the action of zinc ions, high in the electromotive series, does not result in inactivation, but precipitation of the active material is incomplete.

(2) *Organic acids* Several organic acids have been found to precipitate the activity from concentrates: trichloroacetic, tannic, azobenzene-*p*-sulfonic, benzoic, furoic, and salicylic acids. With the first two considerable activity disappears during the isolation of the bacitracin. The results with benzoic acid were variable. Large quantities of furoic acid were required to precipitate the antibiotic and the percentage of recovery was low. Salicylic acid, however, yielded precipitates with all batches tested, the yields were high and no inactivation has been observed.

(3) *Other precipitants* Bacitracin is precipitated from water solution by high concentrations of sodium chloride, acetone, ammonium rhodanilate, Reinecke's salt, and molybdic acid.

*Adsorption* Bacitracin is adsorbed on charcoal, Lloyd's reagent, and aluminum oxide. The problem of elution is being studied.

*Chemical analysis* The most potent preparations prepared in this laboratory by the salicylic acid method and freed from salicylic acid yield negative biuret reactions. After 18 hours' hydrolysis with 6 normal hydrochloric acid, the ninhydrin and Waser color reactions are negative. These negative results suggest that either bacitracin is not a peptide or an impurity obscures the biuret reaction.

Sakaguchi and Folin-Marenzi reactions are negative, before and after hydrolysis, indicating the absence of guanido and phenolic groups in the antibiotic. The optical activity of a preparation assaying 45 units per mg has been determined. The optical density of the solutions made reading difficult. A 1 per cent solution in 0.02 normal hydrochloric acid has a specific rotation at 23°C of  $+5$  degrees ( $\pm 2.5$ ). Preparations from material with a lower unitage per mg had a slightly higher rotation. The optical activity, therefore, may originate in impurities.

The first commercial preparations presented for testing purposes have been found to contain the following impurities: free amino acids (particularly tyrosine), carbohydrates, odorous acidic substances, and pigments.

#### SUMMARY

A progress report on the new antibiotic bacitracin is presented from the point of view of assay, media for production, harvesting, concentration, purification, and chemical properties.

A tentative unit has been defined

An *l*-glutamic acid synthetic medium and a soybean digest medium have been proposed from the point of view of simplicity and uniformity, on the one hand, and high yields on the other

The optimum time for harvesting was found to be after 72 hours of incubation at 37 C

The antibiotic can be extracted from the harvest by *n*-butanol and concentrated by steam distillation *in vacuo*

Pigmented impurities may be removed by magnesium oxide The active principle may be precipitated without loss of potency by salicylic acid

Crude bacitracin, at present available by these methods, is highly soluble and such solutions are stable at 0 to 5 C for 8 months to 1 year It may be lyophilized, and in this dried state, in vacuum vials, remains stable at room temperature

The active principle diffuses through a nitrocellulose membrane, which holds back particles of a molecular weight of more than 2,000 Analyses of the crude product seem to indicate that bacitracin is not a peptide and that the molecule lacks guanido and phenolic groups

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# LYSIS AND LYSIS INHIBITION WITH *ESCHERICHIA COLI* BACTERIOPHAGE

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The present understanding of genetic principles has emerged only after exhaustive study of numerous organisms characterized by various levels of organization. Recently the trend has been toward investigations with the simpler organisms as is illustrated by work with fungi (Beadle, 1945*a*, 1945*b*, Lindegren, 1945) and several of the protista (Moewus, 1940, Sonneborn, 1946). Analysis of the pattern of mutability has made even the bacteria amenable to genetic investigation (Luria and Delbruck, 1943). Genetic studies have, however, been made with organisms at an even lower level of organization than those mentioned above. Luria (1945) with his experiments on host range mutants and Hershey (1946*a*, 1946*b*) with his analysis of plaque size mutants have cleared the way for a more complete inquiry into the genetics of the bacteriophages. Research into the genetics of these organisms may lead to the solution of genetic problems, which other studies have not yet resolved.

Those bacterial viruses which have been most thoroughly investigated genetically belong to a group of seven phages and their mutant forms that comprise the T system (Delbruck, 1946). These phages, named T1, T2, and T7, fall into several subgroups on the basis of serology, electron microscopy, host range, and certain physiological characteristics. One of these subgroups is of particular interest here, the even-numbered phages, T2, T4, and T6. These phages are closely related serologically and show the same characteristic morphology in the electron microscope (Delbruck, 1946).

Hershey (1946*a*, 1946*b*) has shown that they also have another property in common: all three are capable of mutation from the wild type,  $r^+$ , to the  $r$  type. The former type is characterized by the fact that it forms small plaques with very turbid halos on agar plates. The mutant type,  $r$ , forms large plaques with clear halos. Another characteristic distinguishing  $r^+$  from  $r$  type, and probably the basic cause of the plaque size difference, is the time required for lysis of visibly turbid cultures. When highly diluted, suspensions of infected bacteria have the same latent period between infection and lysis whether the phage used for infection be of the  $r^+$  or the  $r$  type. When, however, the infected cultures are visibly turbid, a difference in the latent period occurs. An  $r$ -infected culture will clear between 21 and 30 minutes after infection. In contrast to this, a visibly turbid culture infected with  $r^+$  phage will not clear between 21 and 30 minutes but will

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remain turbid for several hours even though all the bacteria have been infected. This condition will be referred to as "lysis inhibition."

The  $r$  mutations appear to offer a unique opportunity to acquire more information about the difference between a wild type gene and its mutant form as well as about the mechanism of action of a specific gene. Therefore the difference between the  $r^+$  and  $r$  phages was analyzed more closely. Since lysis inhibition seems to be the cause of the difference in plaque morphology, the study of this inhibition was considered the most promising point of attack. The present discussion deals with experiments designed to give a more complete picture of the mechanism of lysis inhibition.

#### METHODS

In the experiments described here the progress of lysis was followed by one of two general procedures: either by measurements of turbidity of infected cultures or by plaque count assays of cultures subjected to various conditions. The turbidity measurements were made with a recently described photoelectric nephelometer (Underwood and Doermann, 1947). The bacteria used were grown in 25-ml cultures made in 0.8 per cent nutrient broth (Difco) containing 0.5 per cent sodium chloride. Cultures were made in tubes, 175 mm by 20 mm, fitted with two-hole rubber stoppers, one hole having a short, cotton-plugged glass tube, the other a long tube reaching to the bottom of the culture and plugged with cotton at its upper end. The latter is for aeration and the former for air escape and for the introduction of phage suspensions. The medium is inoculated with 0.025 ml of a 24-hour aerated broth culture in which the bacteria have grown to saturation. The tube is then rigidly fixed in place in the nephelometer, which is housed in an incubator at 37°C. After inoculation the culture is aerated for 5 minutes to ensure thorough mixing before the first reading is taken. The tube is not removed from the nephelometer during the entire course of the experiment.

In taking a reading, the air flow is diverted into a side arm of the aerating system. Thirty seconds are allowed thereafter before a reading is made. This allows the aeration tube to fill up with the culture and disturbances of circulation to come to rest. With this procedure the readings were found to be reasonably reproducible.

The plaque count procedure was patterned after the one-step growth experiments first described by Ellis and Delbruck (1939). When the plaque count increases in an infected culture, lysis is assumed to be taking place. All the experiments were done with bacteria in the exponential phase of growth. The technique as used here involves several steps, and each step may vary from experiment to experiment. The steps used are as follows: (a) Determining the bacterial titer by colony counts—this serves as a check on the number of infected bacteria expected to be present when multiple infections are made. (b) Addition of phage and a short period of incubation to allow for their adsorption on the bacteria—this will be referred to as the primary infection. (c) Addition of antiphage serum, which will inactivate the unadsorbed phage particles but will not affect the adsorbed ones (Delbruck, 1945a)—it is sometimes feasible and

convenient to omit this step (d) Dilutions in nutrient broth for the purpose of diluting the antiserum to the point of relative inactivity and to reduce the titer of the plaque-forming infective centers to a convenient level for plating (e) Addition of potential inhibitors of lysis in the course of the dilutions in (d)—in many cases these inhibitors are phage suspensions and this step will then be referred to as the secondary infection (f) Plating samples for plaque count at various intervals to determine the degree of lysis

#### RESULTS

*Turbidimetric comparisons of lysis* Presentation of the results obtained by using the foregoing procedures is best begun by a discussion of the turbidity curves shown in figure 1 In all these curves two successive drops of the turbidity may be seen, one of these drops occurring immediately after the addition of the phage, and the other one beginning at the end of the latent period of virus multiplication, i e, 21 to 25 mm after infection In the case of the  $r$  mutants the second drop continues uninterruptedly until the culture is clear This clearing is almost complete within 1 hour after the phage has been added With  $r^+$  strains, however, the second drop is smaller and is followed by a rise and much later by a third drop This third drop was not observed in the case of  $T2r^+$  The last turbidity change brings about what appears to the unaided eye to be clearing

It may be noted here that cultures of  $T2r^+$ -infected bacteria do clear after 5 or 6 hours as judged by visual observation The reason why this is not reflected in nephelometer readings is not clearly understood It is presumed that the bacteria do not break down as completely as they do with the  $r$  mutants As can be seen in figure 1 there is considerable residual scattering with all the  $r^+$  strains Apparently the particles remaining after lysis in  $r^+$ -infected cultures, although too small to give gross turbidity, still retain the property of scattering considerable light These lysates appear somewhat opalescent In  $T2r^+$  lysates this incomplete disaggregation seems to be most pronounced Furthermore, the number of infective centers in filtered  $T2r^+$  broth lysates has in several cases been known to rise by a factor 2 to 3 over a period of several months in the refrigerator (unpublished data of M Delbruck and the writer) This implies that disaggregation of small particles may be taking place The fact that the burst size of  $T2r^+$ -infected bacteria is generally found to be smaller than with those infected with  $T4r^+$  or  $T6r^+$  by a factor of 2 to 3 lends credence to such a hypothesis (Delbruck, 1946)

In order to see whether phage liberation is associated with any of the turbidity changes, plaque count assays were made from a  $T4r^+$ -infected culture in which the turbidity was followed nephelometrically Assays were made by taking samples from the culture by means of capillary pipettes introduced through a side arm in the culture tube With this procedure it was unnecessary to move the tube or to remove the aerating tube from it The results are shown in figure 2, in which the plaque titer and the turbidity are plotted against the same time scale The first point on the plaque titer curve is from an independent

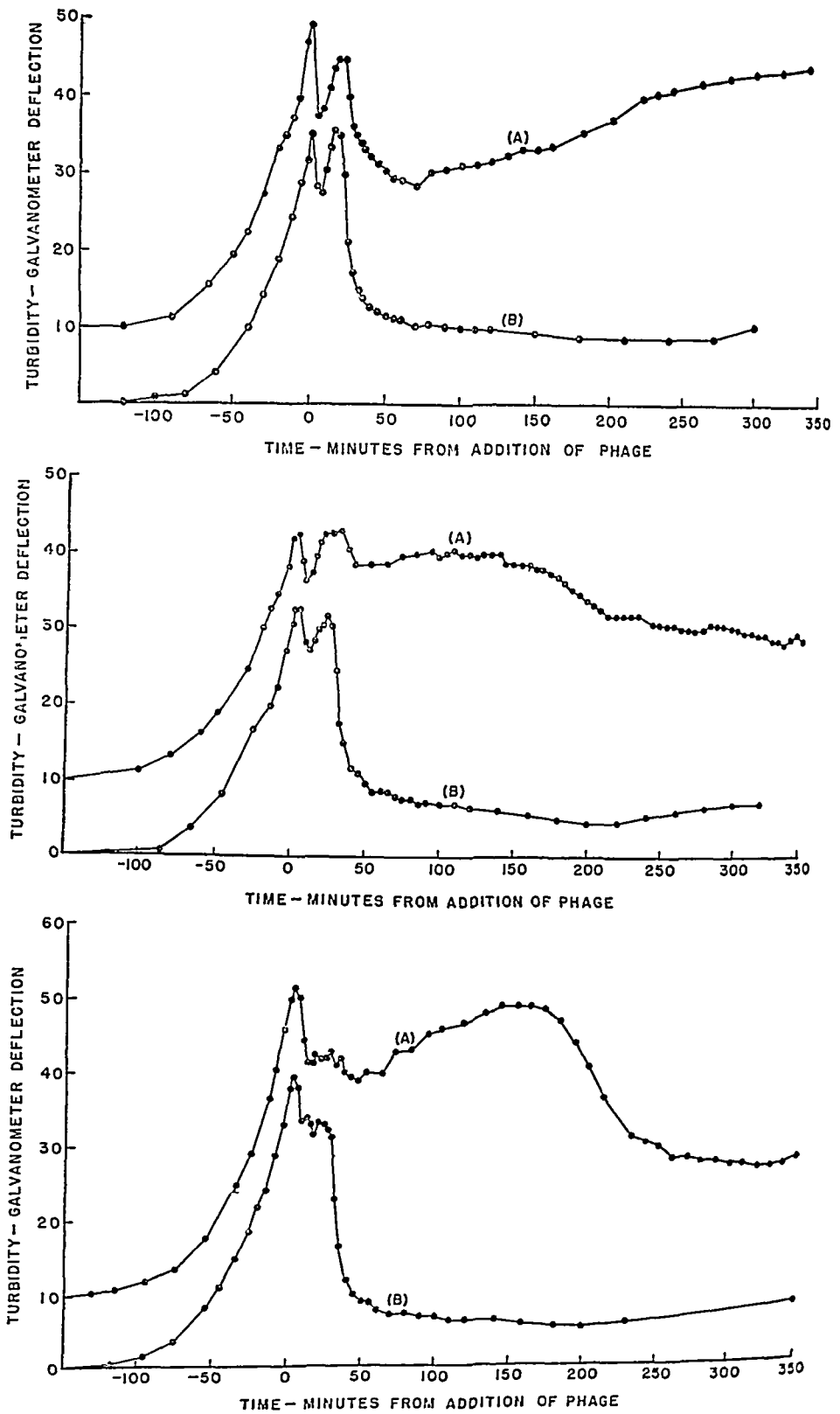


FIG 1

*Top* Turbidimetric comparison of lysis induced by T2r and T2r<sup>+</sup> *Center* Turbidimetric comparison of lysis induced by T4r and T4r<sup>+</sup> *Bottom* Turbidimetric comparison of lysis induced by T6r and T6r<sup>+</sup>

In this figure, curve A represents the turbidity of the r<sup>+</sup>-infected culture and curve B that of the r-infected culture. In all cases curve A is scaled up ten units on the turbidity axis. The multiplicity of infection in these experiments varied from 3.3 to 5.4.

assay of the phage stock used for infecting the culture. The ratio of phage particles to bacteria at the time of infection was approximately 22 to 1.

It is seen in figure 2 that the phage titer dropped slightly in the first 15 minutes after addition of the virus. This is presumably due to the adsorption of several phage particles on single bacteria. At 21 minutes the beginning of a slight rise is noted, which by 30 minutes has reached its maximum with a factor of increase of about 30 over the lowest previous titer. After this time, however, the plaque titer drops again. Apparently only a fraction of the bacteria have lysed and the phage particles liberated from this fraction are being adsorbed on unlysed bacteria. About 50 minutes after this drop, the plaque titer again begins to rise.

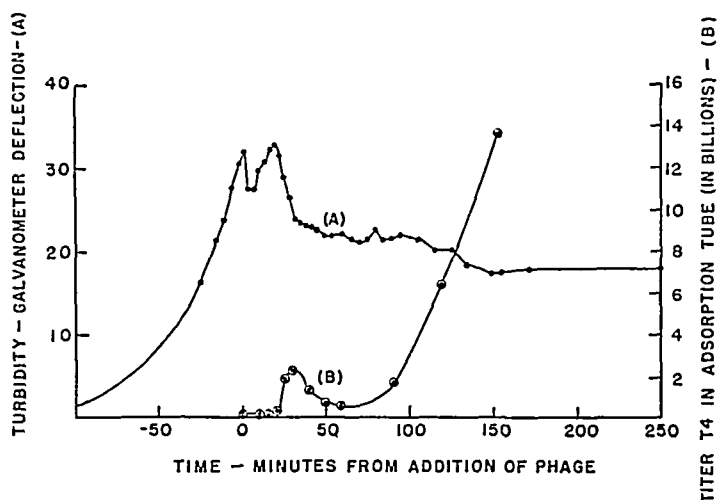


FIG 2 CHANGES IN TURBIDITY OF A  $T4r^+$ -INFECTED CULTURE AS ASSOCIATED WITH THE LIBERATION OF PHAGE PARTICLES

Curve A shows the turbidity of the culture over a period of several hours, whereas curve B shows the changes in phage titer that occurred in the same culture. The multiplicity of infection was about 22.

The first rise in plaque titer occurs at the end of the normal latent period of virus multiplication and corresponds on the time scale to the second turbidity drop. The second rise in plaque titer corresponds with the third drop in the turbidity measurements, which, as pointed out, corresponds to visual clearing. The cause of the first drop on the turbidity curve is at present not clearly understood. Its discussion will be relegated to a later section since it is at this time not relevant to the problem of lysis inhibition.

The second drop in turbidity, which occurs simultaneously with liberation of phage at the end of the latent period, is the criterion of difference between  $r$  and  $r^+$  phages. In infections with  $r$  strains complete lysis occurs at this time, whereas with  $r^+$  strains lysis begins but is presently arrested. In one-step growth experiments (Delbruck, 1940, 1946, Delbruck and Luria, 1942), in which the infected cultures are highly diluted before the end of the latent period, most of the  $r^+$ -infected bacteria do lyse without delay. The failure of the major portion of the

$r^+$ -infected bacteria to lyse in visibly turbid cultures at the end of the latent period must therefore be due to a substance that is released at lysis of the first few bacteria and that inhibits lysis of the remaining ones. Under conditions of the one-step growth experiments lysis of all bacteria would be expected because of the greater dilution of the inhibitor.

*Origin and general properties of the inhibitor* To test the foregoing hypothesis the following experiment was done. A standard culture of bacteria in the exponential growth phase was infected with  $T2r^+$ , the phage being in excess of the

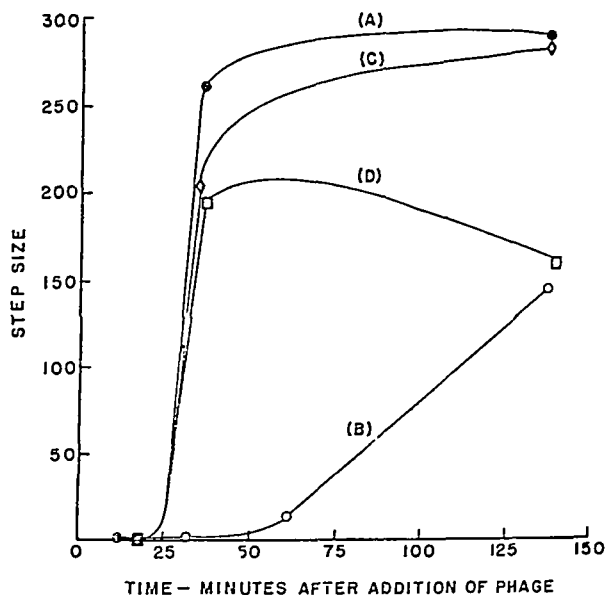


FIG 3 THE RELEASE OF PHAGE PARTICLES FROM  $T4r^+$ -INFECTED BACTERIA AND FROM  $T4r$ -INFECTED BACTERIA EXPOSED TO THE SUPERNATANT FROM AN INHIBITED  $T2r^+$ -INFECTED CULTURE

	Primary Infection	Secondary Exposure
Curve A	$T4r^+$	Broth
Curve B	$T4r^+$	Supernatant
Curve C	$T4r$	Broth
Curve D	$T4r$	Supernatant

bacteria. Thirty minutes later, at the time when lysis inhibition was presumably being effected, the unlysed bacteria were removed by centrifugation. The supernatant was then tested for its ability to inhibit lysis of bacteria previously infected with either  $T4r$  or  $T4r^+$ . The test was made in the manner of the one-step growth experiment described earlier. Separate cultures of bacteria were infected with  $T4r$  and  $T4r^+$  and, omitting step (c), were diluted after adsorption, the final dilution from each being made into supernatant on the one hand and into broth for the control on the other. Platings were made on an indicator strain resistant to  $T2$ , thus making it possible to estimate  $T4$  infection centers exclusively. The results, shown in figure 3, illustrate that the two controls gave normal one-step growth curves. The latent period in both cases ended before 31 minutes. In the case of the cultures diluted in the supernatant, the

latent period of T4 $r$ -infected bacteria was the same as in the controls. In contrast, the T4 $r^+$ -infected bacteria showed only very slight lysis at 31 minutes and only a little more at 61 minutes. Quantitatively it showed less than 2 per cent as many infective centers as the lowest of the other cultures. Several other experiments verified these results. From this experiment it may be concluded that the supernatant contained an agent which can inhibit the lysis of the T4 $r^+$ -infected bacteria but not that of T4 $r$ -infected bacteria.

In order to test whether lysis ultimately occurred because of the breakdown of the inhibitor or whether inhibition was finally overcome in spite of the presence of inhibitor, cleared lysates of T2 $r^+$  were tested for inhibitory power. The tests, conducted in the same way as the previously described tests of the T2 $r^+$  supernatant, showed that lysates do contain inhibitor. Similarly filtrates of cleared T2 $r^+$  lysates were shown to contain inhibitor even after long storage in the refrigerator. In all subsequent experiments, therefore, filtrates were used as inhibitory agent unless otherwise specified.

These results, coupled with those previously described, give rise to the hypothesis that possibly all stock filtrates contain this inhibitor but that only  $r^+$ -infected bacteria are susceptible to the inhibitory action. To test this hypothesis, T1 and T2 $r$  stock filtrates were tested for their ability to delay lysis of T4 $r^+$ -infected bacteria. The experiments were performed like those above, with minor modifications. The T1 filtrate showed no difference from broth and the T2 $r$  filtrate only a very slight effect. T2 $r^+$  filtrate used in precisely the same way again showed inhibitory action. The fact that the T2 $r$  filtrate did show a very slight effect might, if significant, be ascribed to the action of inhibitor produced by T2 $r$ -infected bacteria or it might also be due to the presence of a very small fraction of T2 $r^+$  particles which are always present in T2 $r$  stock because of mutation from  $r$  to  $r^+$  and selection of the wild type (Hershey, 1946a, 1946b).

Thus, for inhibition of lysis it is necessary that the bacteria be infected with an  $r^+$  phage. For marked inhibition it is further necessary that the inhibitor be derived from bacteria lysed by  $r^+$  phage.

*Characterization of the inhibitor.* Several techniques were used in an effort to determine certain characteristics of the inhibitor. In order to obtain some idea of the size of the substance involved, its ability to pass through a cellophane membrane was tested. Five ml of a high titer T6 $r^+$  broth filtrate were placed in a thistle tube whose end was covered with cellophane. The covered end was submerged in five ml of nutrient broth and incubated for 18 hours at 37 C. A sample of the parent T6 $r^+$  filtrate was diluted with nutrient broth by a factor 2 and incubated as a control. The dialyzate was assayed for phage to check against leakage. It was then compared with the incubated filtrate for its ability to inhibit lysis of B infected with T4 $r^+$ . The dialyzate showed no inhibitory effect whatsoever while the control showed decided inhibitory action. It was evident that the inhibitor is not a small, rapidly diffusing molecule.

The estimate of the size of the inhibitor was further limited by testing its sedimentability. A high titer broth stock of T6 $r^+$  was centrifuged at ca 9,000 g for one hour in an angle centrifuge, and the supernatant was removed and

assayed for phage particles. It was found to contain 5.4 per cent of the original titer. This supernatant was compared with a sample of the untreated stock for inhibiting power. T4r<sup>+</sup> on B was the testing agent. Table 1 shows the results as measured by the step size estimated from plaque counts during the expected period of lysis inhibition. It is clear that the inhibitory action was reduced by centrifugation, roughly in proportion to the titer of T6r<sup>+</sup> in the solution tested. Thus the untreated lysate, diluted by a factor 20, showed marked inhibition, almost as much as the undiluted supernatant. The T6r<sup>+</sup> titers were comparable in these cases.

It should be noted here that the low step sizes shown in table 1 cannot be accounted for by the depressor effect described by Delbruck (1945b). This is known from the fact that later platings showed the phage yield to be as large

TABLE 1

*Effect of sedimentation of the virus particles on the inhibitory power of a T6r<sup>+</sup> lysate*

INHIBITOR	TOTAL DILUTION OF INHIBITOR	T6 TITER IN INHIBITION TUBE $\times 10^4$	STEP SIZE*
None, broth control	$\infty$	0	296
Untreated lysate	10	240	3
Untreated lysate	20	120	10
Supernatant	1	130	6
Supernatant	20	6.5	306

A broth stock of T6r<sup>+</sup> was centrifuged at high speed and the supernatant removed and assayed for phage particles. It was found to contain 5.4 per cent of the original T6 titer. The supernatant was compared with the untreated stock for inhibiting power on the lysis of T4r<sup>+</sup>-infected bacteria.

\* Determined between 35 and 40 minutes after the primary infection.

or larger from the inhibited bacteria as from the controls which were not infected secondarily. This consideration also holds true for the experiments described in tables 2 and 3. These are discussed later.

The rough quantitative agreement between the sedimentability of the phage and the inhibitor suggests that the phage and the inhibitor have about the same size and points to the possibility that the phage itself might be the inhibiting principle. This hypothesis was subjected to several tests. First, a refinement of the centrifugation experiment was performed. A sample of phage purified by a differential centrifugation procedure was tested for its ability to inhibit lysis of T4r<sup>+</sup>-infected bacteria. The purified phage was prepared by Dr Seymour Cohen according to the following procedure. Six liters of T2r<sup>+</sup> lysate were centrifuged at 4,000 rpm for 30 minutes. This throws down the large particles of bacterial debris, leaving the phage in the supernatant. The latter was spun at 10,000 rpm for 2 hours, and the pellet taken up in 1 per cent sodium chloride. This suspension was again centrifuged at 4,000 rpm for 1 hour. The phage in the supernatant was sedimented at 10,000 rpm for 2 hours and the



pellet taken up in 100 ml of 1 per cent sodium chloride. The final material contained about  $8 \times 10^{11}$  phage particles per ml (by plaque count assay). For the present experiment a dilution was made in broth to bring the titer to  $3 \times 10^8$ . The latter suspension was used as the inhibitor. The experiment was made in a manner similar to previous experiments and is described in the legend to figure 4. Clearly the purified phage did increase the latent period.

A second test of the hypothesis that the phage itself is the inhibitor is described in table 2, which shows the results of an experiment in which the lysis-inhibiting T6r<sup>+</sup> stock was treated with specific rabbit anti-T6 serum. One aliquot of a filtered T6r<sup>+</sup> stock was incubated for 30 minutes at 37 C with a suitably diluted

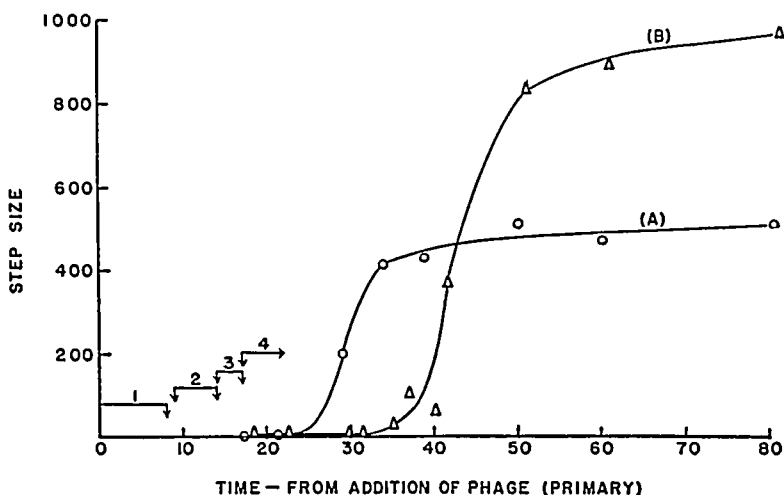


FIG 4 THE EFFECT OF PURIFIED PHAGE IN DELAYING LYSIS OF T4r<sup>+</sup>-INFECTED B

The purification of the phage, based on differential centrifugation, is described in the text. The steps involved in testing this effect are numbered from 1 to 4 above the time scale: step 1, primary infection of B with T4r<sup>+</sup>, step 2, dilution into *broth* (curve A) and into *phage* (curve B) (second infection), step 3, further dilutions, step 4, platings for assays.

antisera. It was then diluted sufficiently to reduce the serum titer to negligible activity and assayed for T6 particles. With T4r<sup>+</sup> on B, the serum-treated T6r<sup>+</sup> stock was compared with an aliquot of the same stock that had been incubated in broth containing no serum. The table shows a close parallelism between phage titer and inhibiting power. This is in agreement with the hypothesis stated above.

A further test made to determine whether the specific properties of the inhibitor agree with those of the phage particles depends on the fact that certain phage-resistant mutants of strain B will not adsorb the phages to which they are resistant (Luria and Delbruck, 1943). For example, the strain B/6, used to assay T4 in the previously described experiments, will not adsorb T6. Therefore, it was reasoned, if the inhibiting agent is the phage itself, T6r<sup>+</sup> mixed with high titer B/6 should lose none of its ability to inhibit lysis, mixing it with B (which will adsorb T6), however, should reduce the inhibiting titer because of

loss of the phage titer. If, on the other hand, some agent other than the phage is responsible for the inhibition, then it seems likely that it should be adsorbed equally well on B and on B/6. To test these alternatives the experiment described in table 3 was made. It can be concluded from this experiment that contact with B reduced the inhibiting potency of the T6r<sup>+</sup> stock, whereas contact with B/6 did not. Within the limits of error of such an experiment the reduction of inhibiting power was proportional to the reduction of the phage titer.

Another result that would be expected if the phage itself is the inhibiting agent is this: if B/6 were infected with T4r<sup>+</sup>, then lysis of this system should be inhibited in the highly diluted growth tube by the presence of T2r<sup>+</sup> since it can be adsorbed on such a system, the presence of a T6r<sup>+</sup> lysate, however, should have

TABLE 2

*Effect of treatment with specific antiphage serum on inhibiting power of a filtered T6r<sup>+</sup> lysate*

INHIBITOR	TOTAL DILUTION OF INHIBITOR	TITER OF ACTIVE T6 IN INHIBITION TUBES $\times 10^3$	STEP SIZE*
None, broth control	$\infty$	0	193
Untreated lysate	100	380	9.6
Untreated lysate	400	95	33.5
Untreated lysate	1,000	38	126
Untreated lysate	4,000	9.5	218
Treated lysate	10	260	10.4
Treated lysate	100	26	231

An aliquot of a T6r<sup>+</sup> lysate was incubated at 37 C with a suitably diluted anti-T6 serum. The factor of inactivation was 14.3. The treated portion and an untreated portion were compared for their ability to inhibit lysis of B by T4r<sup>+</sup>.

\* Measured between 35 and 40 minutes after the primary infection. Later platings showed that in all cases a step size of 200 or greater was attained.

no effect on the time of lysis. The experiment made to check this prediction is outlined in figure 5. It is seen that lysis of the system T4r<sup>+</sup> on B/6 was not inhibited in either broth or T6r<sup>+</sup> suspension. The T2r<sup>+</sup> did, however, increase the latent period, showing the system to be susceptible to inhibition.

From the last two experiments described it seems clear that one common property of phage and inhibitor is the specificity of host range, for the inhibitor will act only on a host capable of adsorbing the phage and is removed from the filtrate by adsorption only on such bacteria as are capable of adsorbing the phage. A second common property is that of specificity of reaction with anti-phage serum. Inhibitor and phage are inactivated at the same rate by specific anti-phage serum. A third characteristic common to both is that of size. Neither inhibitor nor phage will pass through a cellophane membrane, but both are sedimented at the same rate in the centrifuge. These results demonstrate that the cause of lysis inhibition is the secondary adsorption of an r<sup>+</sup> phage.

*Factors influencing the inhibition of lysis* With the phage thus shown to be the inhibitor, the next problem studied concerned the number of phage particles adsorbed per bacterium in the secondary infection necessary for a delay of lysis. In order to set a lower limit on the estimate, an experiment was performed in which T4r<sup>+</sup>-infected bacteria were exposed to T2r<sup>+</sup> at several concentrations for a period of 5 minutes in the middle of the T4 latent period. Four simultaneous one-step growth curves of T4r<sup>+</sup> on B were made. The procedure was to mix a rapidly growing culture of B ( $1.6 \times 10^8$  bacteria per ml) with about 10 T4 particles per bacterium. Two minutes were allowed for adsorption, and then a

TABLE 3

*Inhibiting power of T6r<sup>+</sup> lysate after exposure to sensitive and to resistant bacteria*

INHIBITOR	TOTAL DILUTION OF INHIBITOR	TITER OF T6 IN INHIBITION TUBE $\times 10^4$	STEP SIZE*
None, broth control	$\infty$	0	186
Untreated lysate	100	400	12 1
Untreated lysate	400	100	26 8
Untreated lysate	2,000	20	240
Exposed to B/6	80	450	10 5
Exposed to B/6	320	112	25 5
Exposed to B/6	1,600	22	244
Exposed to B	40	61	24 8
Exposed to B	130	19	336

Aliquots of a T6r<sup>+</sup> lysate were added to cultures of B and of B/6. The bacterial cultures were standard 150 minute cultures in the exponential phase of growth and at a titer of about  $5 \times 10^7$  bacteria per ml. The phage lysate was added in the volume ratio of 1:40 giving a titer of about  $10^9$  particles per ml. Adsorption on B was allowed to proceed for 4 minutes, at the end of which time the culture was poured on a sterile, sintered, glass filter. Adsorption on B/6 was allowed to proceed for 11 minutes before filtration was begun. Filtration in both cases was completed in 2 minutes and 30 seconds. The filtrates were assayed twice for their phage titer and then tested for their ability to inhibit lysis of T4r<sup>+</sup>-infected bacteria.

\* Measured between 35 and 40 minutes after the primary infection.

dilution by a factor 10 was made into specific anti-T4 serum. This reduced the titer of unadsorbed T4 to a level which would not interfere with the significance of the results. After allowing 7 minutes for serum action, a dilution by a factor 40 was made into broth as a control and into three concentrations of T2r<sup>+</sup> as experimental. The titers of T2 were, respectively,  $6 \times 10^7$ ,  $3 \times 10^8$ , and  $1.5 \times 10^9$  particles per ml. Five minutes were allowed for this secondary infection, then each culture was diluted by a factor 100 in broth, and platings from these tubes were made against an indicator strain resistant to T2. Further dilutions were made from these tubes for convenience in later post-burst platings. Platings were made at intervals and the results are seen in figure 6. In all cases the growth curves from the experimental cultures show a longer latent period than

do those from the controls. In a similar experiment using the same T2 stock at a still higher dilution ( $1.2 \times 10^7$  particles per ml) no inhibition was noted. The growth curve followed the control almost exactly.

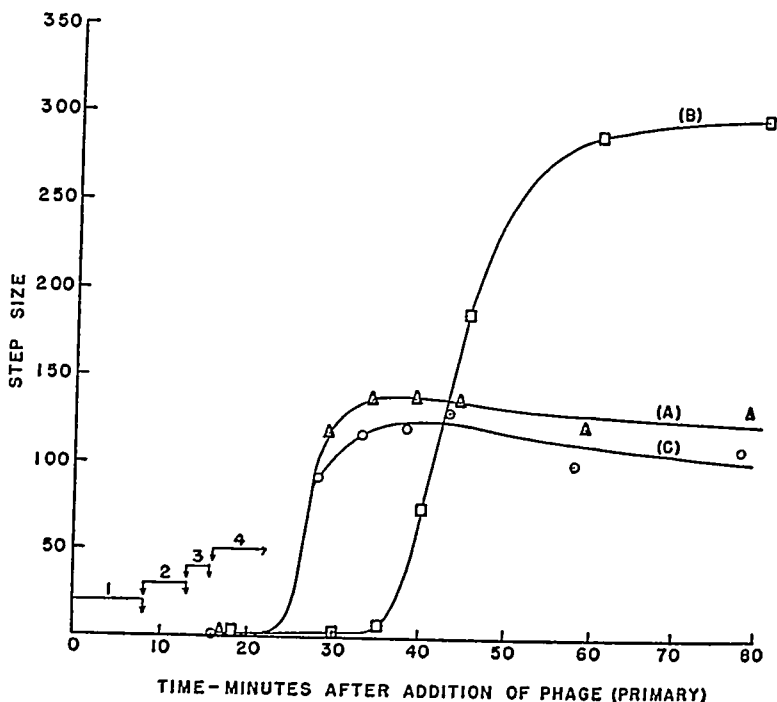


FIG 5 EXPOSURE OF B/6 INFECTED WITH T4r<sup>+</sup> TO A SECONDARY ADDITION OF T6r<sup>+</sup> IN CONTRAST TO A SECONDARY ADDITION OF T2r<sup>+</sup>

B/6 is known to be incapable of adsorbing T6 but adsorbs T2 rapidly. The steps involved in testing whether T6r<sup>+</sup> lysate is capable of inhibiting lysis of T4r<sup>+</sup>-infected B are numbered from 1 to 4 above the time scale in the figure. Step 1 shows the time allowed for the primary adsorption of T4r<sup>+</sup>. From the adsorption tube a dilution was made into broth for the control (curve A), into a suspension of T2r<sup>+</sup> (curve B), and into a suspension of T6r<sup>+</sup> (curve C). Step 2 indicates the time allowed for the secondary exposure. Step 3 involves further dilutions for convenience in plating, and step 4 indicates the period over which platings are made. These platings determine the points on the curves. The purpose of curve B (secondary infection by T2r<sup>+</sup>) is to show that the system B/6 infected with T4r<sup>+</sup> is capable of being inhibited.

Thus the lower limit of inhibition in these experiments was reached in the range  $1.2$  to  $6.0 \times 10^7$  particles per ml. The number of phage particles adsorbed under these conditions may be estimated as follows. The amount of T2 adsorbed may be calculated from the expression

$$N/N_0 = e^{-k_B t} \quad (1)$$

in which  $1 - N/N_0$  is the fraction of phage adsorbed,  $B$  is the bacterial titer,  $t$  is the time of exposure (in minutes), and  $k$  is the adsorption rate constant characteristic for each phage-bacterial system. The value of  $k$  may be estimated from experiments with higher concentrations of  $B$ . Thus, when T2 is added to a culture of rapidly growing bacteria at  $5 \times 10^7$  organisms per ml, the fraction of particles adsorbed in 5 minutes is between 75 and 95 per cent. Substituting

these values in equation (1) we find that  $k$  for our system lies between the values  $5.5 \times 10^{-9}$  and  $1.1 \times 10^{-8}$ . Again using equation (1) and assuming  $k$  to be between the limits just found, we can calculate the number of particles adsorbed in the inhibition experiment. For a T2 concentration of  $6 \times 10^7$  we thus find this average multiplicity of secondary infection to be between 1.6 and 3.5. The conclusion drawn from this experiment is that one, or at most very few, phage particles are capable of delaying lysis of an infected bacterial cell.

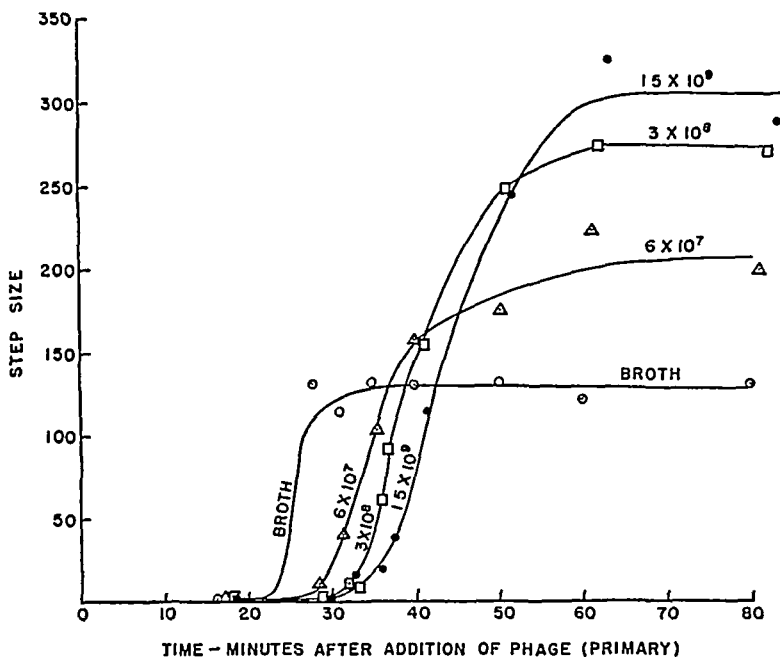


FIG. 6. DETERMINATION OF THE LOWER LIMIT FOR THE NUMBER OF PHAGE PARTICLES WHICH MUST BE ADSORBED ON A BACTERIUM TO DELAY LYSIS.

This figure shows the inhibition curves obtained when a  $T4r^+$ -infected B culture was exposed to three concentrations of  $T2r^+$  as compared to the control curve where no secondary infection was made. The details of the experiment are described in the text. For the method of determining the average number of phage particles secondarily adsorbed per bacterium, see text.

The number written on each curve shows the titer of T2 per ml in the secondary adsorption tube.

If inhibition is caused by a secondary adsorption of  $r^+$  phage particles and can be effected even though the average multiplicity is as low as 3.5, then one would expect inhibition of lysis in cases in which a sufficient excess of phage particles is given in the primary infection. To test this correlation, four simultaneous one-step growth experiments were made with different multiplicities of  $T4r^+$  infection. The multiplicities used were 0.4, 4, 10, and 33. Five minutes were allowed for adsorption and during this period 94 per cent of the virus particles were adsorbed. Then a dilution of 40 (20 in the case of 0.4) was made into antiserum of sufficient titer to reduce the unadsorbed phage by a factor  $8 \times 10^3$  in 10 minutes. After the period of serum action a dilution of 500 was

made for the first growth tube. Platings were made from this tube and from subsequent dilutions.

The results, seen in figure 7, show that marked inhibition occurred only in the tube with the highest multiplicity, which was 33. The 10-fold infection showed none, in spite of the fact that, on the average, each bacterium adsorbed 9.4 phage particles. This suggests that the primary and secondary infections must be separated by several minutes. Some estimate of this separation may

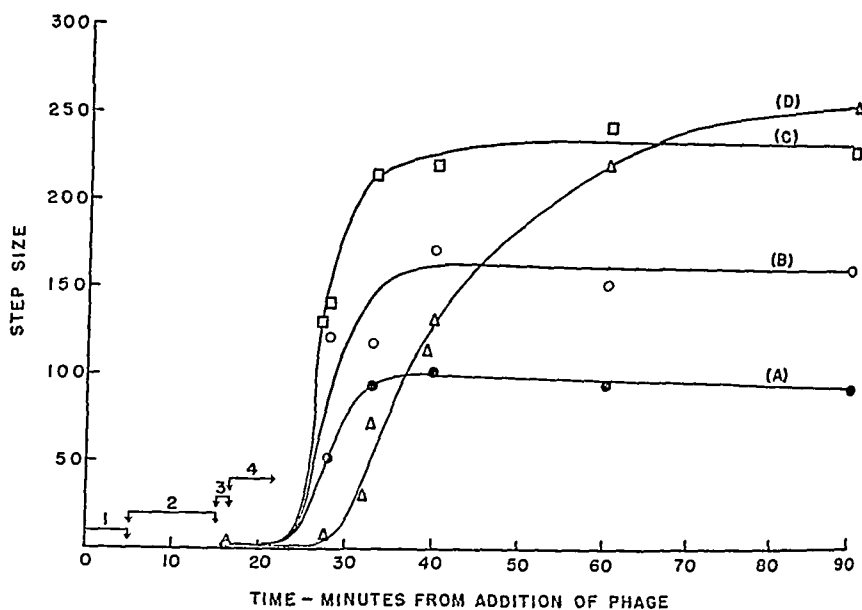


FIG 7 THE EFFECT OF THE MULTIPLICITY OF PRIMARY INFECTION ON THE LATENT PERIOD OF  $r^+$ -INFECTED BACTERIA

Four cultures of B were infected with the following average multiplicities of  $T4r^+$ , respectively 0.4 (curve A), 4.0 (curve B), 10 (curve C), 33 (curve D). Five minutes were allowed for adsorption (step 1). Then a dilution was made into appropriately diluted antiserum to stop further adsorption and to inactivate unadsorbed phage particles (step 2). Dilutions were made from the antiserum tubes (step 3), and platings were made to determine the duration of the latent period and the step size (step 4).

be obtained by determining how many particles will be adsorbed in each minute of adsorption. With equation (1) again, the number of phage particles adsorbed per bacterium in each successive minute was calculated and the results tabulated

TIME INTERVAL (MINUTES)	% ADSORBED PER INTERVAL	AVERAGE ADSORBED PER BACTERIUM PER MINUTE		
		4X	10X	33X
0-1	43	1.7	4.3	14.2
1-2	24	1.0	2.4	7.9
2-3	14	0.6	1.4	4.6
3-4	8	0.3	0.8	2.6
4-5	5	0.2	0.5	1.6
0-5	94	3.8	9.4	20.9

We have seen that 3 phage particles adsorbed secondarily are sufficient to effect inhibition. Since in the 10-fold multiplicity there were still 5.1 particles to be adsorbed after the first minute and no inhibition occurred, we can conclude that more than 1 minute must separate the primary and secondary infections. Judging from the multiplicity of 33 which did show inhibition, however, the secondary infection could come as little as 2 or 3 minutes after the primary infection.

Several experiments were also done to test at what time an infection with a heterologous  $r^+$  phage could induce lysis inhibition. After primary infection with  $T4r^+$  secondary infections were made with  $T6r^+$ . In several experiments 2-minute exposures were made at the following times: 4.5, 10.0, 12.5, 14.0, and 18.0. In all cases lysis was delayed from 20 to 30 minutes over that in the controls. The results did not show a significant difference in the effectiveness of the secondary infection as a function of the time when it was applied. In another experiment 1-minute exposures were made at 1.5 minutes and at 5.0 minutes. In both of these cases inhibition was clearly evident. To test closer intervals than 1.5 minutes is technically difficult, and the results are difficult to interpret because of the "depressor effect" previously described by Delbruck (1945b).

*Investigation of the first drop in turbidity* The cause of the initial drop in turbidity seen in all the nephelometric curves in figure 1 is, at present, not clearly understood. It could be due to lysis of bacteria, to some alteration in the light-scattering properties of the newly infected bacteria, or to both of these causes. The results shown in figure 1 show only that it is common to all six phage-bacterial systems used in these experiments. Other experiments have shown that it occurs also with T5 but not with T1 (Underwood and Doermann, 1947), T3, or T7. Assays of the number of infected bacteria during the latent period and comparison of this number with the number of bacteria present before infection (from colony count assays) showed little or no loss of infective centers, and hence this dip would not be thought to be due to lysis of the bacteria. This criterion is not very reliable, though, since the magnitude of the difference is not large and since other factors cannot be controlled. One such factor is the division of bacteria in the first few minutes after virus addition and before they have actually adsorbed a phage particle. Another factor is the possibility that infected bacteria might be capable of dividing. Either of these factors might compensate for the infective centers lost by lysis.

The question of the interpretation of the first drop in turbidity was also approached from a slightly different angle. Turbidity measurements were made of cultures of bacteria infected with varying amounts of  $T6r$ ,  $T6r^+$ , and  $T4r^+$ . Several facts can be determined from a study of figure 8, which shows the curves obtained with infections of various multiplicities. In the case of  $T4r^+$  it is noted that the initial dip with a multiplicity of 3.6 is only slightly smaller than with multiplicities of 13 and 16. Also it is seen that the recovery from the dip proceeds at a slower rate as the multiplicity increases. This could readily be interpreted if one assumes that the character of the bacterial surface changes

because of the adsorption of phage particles and that one or a few phage particles are about as effective as 10 to 15 in bringing about this change. Furthermore, one must assume that after most of the phage particles have been adsorbed, recovery of the original surface takes place. One would expect this recovery to be delayed considerably at such high multiplicities as 61, where adsorption continues over a longer period of time. This is seen to be the trend in figure 8-A.

The curves showing the effect of various multiplicities of T6r and T6r<sup>+</sup> show several notable differences, however. Except at the lowest multiplicities, T6r

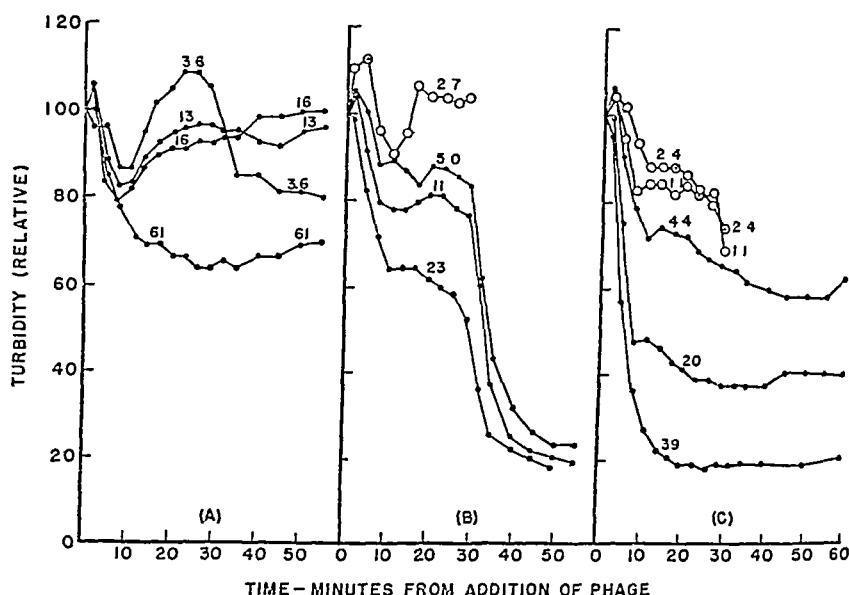


FIG 8 THE INITIAL DROP IN TURBIDITY AS A FUNCTION OF THE AVERAGE MULTIPLICITY OF INFECTION

(A) infection with T4r<sup>+</sup>, (B) infection with T6r, (C) infection with T6r<sup>+</sup>

The multiplicities of infection are written with each curve. The encircled points have been corrected for the uninfected bacteria present, on the assumption that these bacteria maintain their normal exponential growth rate.

The turbidity is shown from the time of addition of the phage suspension, and for the sake of comparison all turbidities were at this time arbitrarily adjusted to 100. The absolute bacterial titers were  $5$  to  $10 \times 10^7$  B per ml which is in the exponential phase of growth. All infections with a given phage were made from the same stock filtrate.

induces a larger dip than T4r<sup>+</sup> for comparable phage-bacterial ratios. T6r<sup>+</sup> produces an even larger dip than T6r. Another difference, particularly noticeable in figure 8-C, is that the recovery is absent in T6r<sup>+</sup>-infected bacteria. These experiments then suggest that the dip is due to an irreversible loss and probably due to lysis of a fraction of the bacteria. Emphasis for this suggestion comes from the 80 per cent drop in turbidity at a multiplicity of 39 with T6r<sup>+</sup>. Examination of this tube at 20 minutes showed it to be completely clear so far as the naked eye could see. Thus, in T6 infections one would be inclined to suspect lysis from without (Delbruck, 1940) as the cause of part of the initial dip, whereas with T4r<sup>+</sup> some reversible process seems to cause it.



Lysis from without is probably not the sole cause of the dip even in the T6 curves, since, with a multiplicity of 2.7 of T6r, a recovery is very obvious even when the curve is corrected for uninfected bacteria. With the two lowest multiplicities of T6r<sup>+</sup> (1.1 and 2.4), the lower of the two shows a slightly greater drop than does the higher multiplicity. This is not in agreement with the general trend of the rest of the T6 curves in which the amount of lysis from without is directly related to the multiplicity of infection. These two considerations point to the likelihood that there is an initial dip even here that is not caused by lysis of bacteria. The most satisfactory explanation of the initial dip appears to be some change in the light-scattering properties of the infected bacteria, but high multiplicities of infection induce an additional drop that is due to lysis of a fraction of the bacteria.

One further indication that the initial dip in turbidity is due to a change in all the bacteria comes from experiments with T5. It is known that certain phages require that calcium be present in the medium in order for them to complete a cycle of infection, reproduction, and lysis. T5 has this requirement. Calcium is, however, not required for the growth of *E. coli* strain B nor for the adsorption of T5 on the host. When bacteria are infected with T5 in a medium in which calcium is available, a dip and subsequent recovery occur and lysis of the bacteria follows at the expected time (Underwood and Doermann, 1947). However, when T5 infection takes place in a medium where no calcium is available (in these experiments with oxalate present), the dip takes place, but neither recovery nor lysis are observed. It appears from this that recovery from the dip depends on some system which requires calcium to reverse the cause of the dip, rather than on cell division to compensate for the loss of a fraction of the bacteria.

#### DISCUSSION

The results described above present the following picture of the actual events occurring under conditions of lysis inhibition. Bacteriophages of the r<sup>+</sup> type infect bacteria that reproduce the phage during the latent period, at the end of which lysis begins, first in a few bacteria, but increasing rapidly. Immediately after liberation of the first new phage particles they become reabsorbed on those bacteria which have not lysed. The reactions involved in and following this secondary adsorption of an r<sup>+</sup> phage delay the lysis of the secondarily infected bacteria.

The results of growth tube experiments have, however, never duplicated the long delay in lysis shown by nephelometric determinations. The nephelometer experiments indicate a delay of 75 to 150 minutes while growth tube experiments show delays of 15 to 40 minutes. This difference may be due to the fact that the situation in the growth tube is not quite analogous to that in the adsorption tube. First, in growth tube experiments it is not experimentally feasible to make a homologous secondary infection as late in the latent period as it occurs in the adsorption tube, since to wait until some bacteria burst would make it impossible to distinguish between the effects of the products of the burst and

the phage added from the outside. Furthermore, in the adsorption tube a self-regulatory system exists in that inhibitor is produced only if and when the inhibition begins to break down. This type of system could promote extended delay of lysis, since bacteria would lyse until a secondary infection became sufficient for stopping lysis, and more bacteria would lyse only to the extent of maintaining a sufficient level of secondary infection. It is not feasible to imitate this situation in growth tube experiments.

The difference between the results of the two techniques may also be due to the fact that they measure two different things. It is very difficult to determine from turbidity measurements the exact time when the inhibition of lysis begins to break down in the adsorption tube, since this drop in turbidity is a very gradual one. On the other hand, the growth tube experiments measure the beginning of phage liberation more accurately. It might also be noted that in at least one experiment in which both the primary and secondary infections were made with the same phage ( $T4r^+$ ), the inhibition did last longer. Perhaps there is a difference in the effect of heterologous and homologous secondary infections.

Considering these conditions and differences it is not unreasonable to make the assumption that the condition of lysis inhibition in the adsorption tube is an extension of the phenomenon observed in the growth tubes.

Although the experiments discussed here reduce the phenotypic difference between  $r^+$  and  $r$  from a mass culture effect to one concerning individual bacteria, nevertheless they do not elucidate the mechanism underlying the difference. It is clear that the mechanism involves the new phages to be liberated or some by-product of their production. It is also seen that the type of phage which is secondarily adsorbed determines whether or not lysis will be delayed. It might then be said that both of these reactions are type-specific since they both depend on the type of phage involved. Thus it is requisite that the bacteria be both primarily and secondarily infected with the  $r^+$  type for inhibition of lysis. But we have no clue from these experiments as to what  $r^+$  accomplishes inside the bacterium that the  $r$  does not accomplish or vice versa. And we do not know what  $r^+$  prevents from happening when it is secondarily adsorbed.

Even though these results do not explain the mechanism, they do point to several types of experiment that might be done in an effort to learn more about it. For we can now break the mechanism down into effects caused by the primary infection and those caused by the secondary infection. Since it is known, for example, that the secondary infection is one of the determining factors, it can be investigated further using mutant phages which require adsorption cofactors (Anderson, 1945). Early experiments with synthetic media gave complicated results, presumably because of the lack of adsorption cofactors in the medium, but now, with additional experiments, we may be able to interpret them. It might also be possible to duplicate the phenotypic difference by exposing the infected bacteria to specific respiratory poisons shortly before lysis. In this way one might learn something about the chemical nature of the reactions involved. Still another approach might be that of treating the phages to be

adsorbed secondarily in an attempt to remove the inhibition property from the  $r^+$  phages or to add it to the  $r$  phages

One further point that deserves mention is the fact that there is a decided increase in the number of phage particles liberated per bacterium after lysis inhibition has been effected. Figures 4, 5, and 6 show that the step size is higher when  $T4r^+$ -infected bacteria are inhibited than when they are not inhibited. Hershey (1946a) has noted that  $T2r^+$  has a selective advantage in cases in which it is competing with  $T2r$  for survival in mixed cultures. This is true in spite of the fact that the mutation rate from  $r^+$  to  $r$  is considerably higher than is the reverse rate. Since  $r$ -infected bacteria do not respond to lysis inhibition, the  $r^+$  burst size becomes effectively larger than the  $r$  burst size. Whether this difference is an important factor in maintaining the wild type cannot be decided without closer analysis, but the results are suggestive of that hypothesis.

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#### SUMMARY

Lysis caused by  $r^+$  and by  $r$  strains of the even-numbered phages active on *E. coli* strain B is studied both by means of plaque count methods and by turbidimetry. From the turbidimetric studies it is seen that one characteristic common to all the lysis curves discussed is an initial dip in turbidity, which occurs immediately after addition of the phage. Present evidence indicates that it is due to a reduction and recovery in the light-scattering properties of bacteria resulting from the phage infection. With high multiplicities of infection immediate lysis of a fraction of the bacteria is also induced, and this adds to the magnitude of the initial drop. A second drop occurs in all cases at the end of the normal latent period. This drop is, however, quite different with  $r^+$  infection from what it is with  $r$  infection. With the  $r$  phages it continues to complete clearing, but with the  $r^+$  phages it is small and is succeeded by a rise in the curve. This difference is the basic difference between  $r^+$  and  $r$  infections, and it has here been called lysis inhibition. After the period of inhibition a third drop in turbidity occurs, except with  $T2r^+$ , and this drop represents complete clearing.

The further analysis of lysis inhibition has yielded the following picture: the phenomenon requires that a bacterium be infected twice by  $r^+$  phages of the same or similar type; the second infection, if it is an infection by the same type of phage as the first, may come any time after the primary infection has been established, which takes about 3 minutes; present evidence indicates that a second infection by only a single phage particle will accomplish the inhibition.

Lysis inhibition has the effect of increasing the burst size.

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# THE EFFECT OF SEVERAL COMPOUNDS ON THE INHIBITION OF BACTERIAL GROWTH BY SULFAGUANIDINE, SULFAMERAZINE, AND SULFASUXIDINE<sup>1</sup>

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Sulfaguanidine, sulfamerazine, and sulfasuxidine have been found to be particularly effective in preventing the growth of the gram-negative intestinal bacilli. Each compound has a different N<sup>1</sup> substituted group. Berkman and Koser (1943) have shown that sulfapyridine interferes with nicotinamide-stimulated respiration of a dysentery bacillus. It has been shown that sulfathiazole inhibits the carboxylase activity of *Staphylococcus aureus* (Sevag *et al.*, 1945). Both of these inhibitions are thought to be due to interference by the N<sup>1</sup> substituted portion of the sulfonamide molecule with a structurally similar part of a coenzyme molecule. Sevag and Green (1944) have shown that sulfonamides interfere with the metabolism of *S. aureus* by preventing the synthesis or utilization of the essential amino acid, tryptophane. *Para*-aminobenzoic acid (PABA) reverses the action of almost all of the sulfonamides and is thought to do so because of its structural similarity to the parent molecule, sulfanilamide. It competes with the sulfonamide for an active site on an enzyme of the bacterial cell. Since it acts as an inhibitor of growth, Sevag, Henry, and Richardson (1945) suggested that it reverses the sulfonamide action by successfully competing for the enzyme but is itself much less inhibitory than the sulfonamide. *Para*-aminobenzoic acid has been shown to be a growth factor for a few organisms. Comprehensive reviews on this subject and on the mode of action of the sulfonamides have been published by Henry (1943) and by Sevag (1946).

The preceding statements indicate that, if any physiologically active compound reverses the inhibition of sulfaguanidine, sulfamerazine, or sulfasuxidine, it is either a growth factor, the utilization or synthesis of which is being interfered with by the particular sulfonamide, or it is so closely related structurally to some part of the sulfonamide molecule that it is competing with it for an active site on the enzyme that takes part in normal metabolism. It was thought that compounds that (1) were parts of coenzyme molecules or contained parts of these molecules, (2) were amino acids necessary to growth, or (3) were structurally similar to part of the sulfonamide molecule would be interesting to investigate in relation to their effect on the bacteriostatic action of the sulfonamides. Such an investigation might yield further information about the mode of action of these sulfonamides, and it might reveal a synergistic combination worthy of therapeutic trial.

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The experimental work here presented was designed to determine the effect of several compounds on the rate of growth, the glucose metabolism, and the sulfonamide bacteriostasis of *Escherichia coli* and *Salmonella paratyphi* B. Their effect on these organisms, in the absence of sulfonamides, was determined first, since this often gives a clue as to the type of action being exerted. The two organisms were chosen because both are gram-negative intestinal bacteria with different biochemical activities, and both could be grown in a simple synthetic medium. Several of the compounds have not been previously tested for their effect on the growth of *E. coli* and none of them on *Salmonella paratyphi* B. Their effect on the bacteriostatic action of the three sulfonamides likewise has not been previously reported.

#### EXPERIMENTAL METHODS

**Organisms** The *E. coli* and *S. paratyphi* B were stock cultures from our own laboratory. They gave typical biochemical reactions. These organisms were inoculated into nutrient broth, and the 24-hour growth was centrifuged and washed twice in the synthetic medium, without the glucose, given below. The washed organisms were suspended in 5 ml of this medium used in the growth determinations.

**Measurement of effect on growth** The compounds listed in table 1 were dissolved in redistilled water and autoclaved, with the exception of thiamine, which was sterilized by filtration. Each was added to 4 ml of the inoculated growth medium in the concentrations of 2 micrograms per ml and 100 micrograms per ml. The growth medium had the following composition:

Sodium ammonium hydrogen phosphate	1.5 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulfate	0.2 g
Glucose	5.0 g
Redistilled water	1,000.0 ml

The final volume was adjusted to 5 ml with redistilled water. The tubes were incubated at 37 C and growth was determined by observing the turbidity at intervals.

The sulfonamide solutions were sterilized by being placed in boiling water for 10 minutes. The concentration of each that would just prevent growth in 24 hours was used in all tests. Tubes were set up, and inhibition or reversal of inhibition in the presence of the 19 compounds previously tested for the effect on growth in the absence of sulfonamides was determined by observing the turbidity. Controls with no added test substances were included with each experiment. All tests were set up in triplicate, and results were considered positive only when all three tubes in each test were positive. Results shown in the tables are derived from at least three separate determinations.

**Measurement of acid production and glucose utilization** Twenty ml of medium containing the compounds that had been found to effect growth were used. The sulfonamides were added in the concentrations used in the growth determinations. One ml of each culture was removed after 72 hours' incubation, diluted

TABLE 1

*Effect of several compounds on the growth of E coli and S paratyphi B with and without sulfonamides*

COMPOUNDS TESTED	CONC μg/ml	S PARATYPHI B				E COLI		
		No sulfa	0.05 mg/ml sulfamer	0.05 mg/ml sulfaguan	0.05 mg/ml sulfasux	No sulfa	0.005 mg/ml sulfamer	0.1 mg/ ml sul faguan
Thiamine	2	0	0	0	0	0	0	0
	100	—	0	—	—	0	0	0
Riboflavin	2	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
Nicotinic acid	2	+	0	0	0	0	0	0
	100	+	0	0	0	0	0	0
Pyridoxine	2	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
Mix of 4 above	2	0	0	—	0	0	0	0
	100	—	—	—	—	0	0	0
Choline	2	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
Calcium pantothenate	2	+	0	0	0	0	0	0
	100	+	0	0	0	0	0	0
Inositol	2	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
PABA	2	0	+	+	+	0	+	+
	100	—	+	+	+	—	+	+
Urea	2	+	0	0	0	0	0	0
	100	+	0	0	0	0	0	—
Uric acid	2	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
Caffeine	2	+	0	0	0	0	0	0
	100	+	0	0	0	0	0	0
Nembutal	2	0	0	0	0			
	100	+	0	0	0			
Uracil	2	0	0	0	0	0	0	0
	100	+	0	0	0	0	0	0
Allantoin	2	0	0	0	0	0	0	0
	100	0	0	—	—	0	—	—

TABLE 1—Continued

COMPOUNDS TESTED	CONC μg/ml	S. PARATYPHI B				E. COLI		
		No sulfa	0.05 mg/ml sulfamer	0.05 mg/ml sulfaguan	0.05 mg/ml sulfasux.	No sulfa	0.005 mg/ml sulfamer	0.1 mg/ ml sulfa
Hydantoin	2	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
Creatine	2	0	0	0	0	—	0	0
	100	+	0	0	0	—	0	0
Asparagine	2	0	0	0	0	+	0	+
	100	+	+	+	+	+	+	+
l-Cystine	2	+	+	+	+	0	+	+
	100	+	+	+	+	+	+	+
l-Tryptophane	2	+	0	0	0	0	0	+
	100	+	+	+	+	+	+	+

0 = neither stimulation nor inhibition, — = inhibition of growth or enhanced inhibition, + = stimulation of growth or reversal of sulfa action, results determined by visible turbidity

to 100 ml with distilled water, and titrated with N/10 NaOH to determine the acid produced

To determine the glucose utilized, 5 ml of culture medium were removed after 72 hours' incubation and diluted to 100 ml in a volumetric flask. The glucose, in 5 ml of this diluted solution, was determined by the Shaffer-Hartmann method as modified by Stiles, Peterson, and Fred (1926).

#### RESULTS

Only a few of the compounds had any growth-stimulating or growth-inhibiting effects on *E. coli* or on the action of the sulfonamides. Table 1 shows that PABA inhibited growth in a concentration of 100 micrograms per ml and creatine inhibited growth in a concentration of 2 micrograms and 100 micrograms per ml. Asparagine, cystine, and tryptophane were the only substances which stimulated growth. One hundred micrograms per ml of these amino acids increased the amount of acid produced from glucose and the glucose utilized by *E. coli*. Urea in a concentration of 100 micrograms per ml increased the inhibition of growth by sulfaguanidine, and allantoin increased the bacteriostatic effect of both sulfamerazine and sulfaguanidine. Asparagine, cystine, and tryptophane reversed the inhibition by sulfamerazine and sulfaguanidine for *E. coli*.

A more detailed study was made with *Salmonella paratyphi* B. Two micrograms per ml of nicotinic acid, calcium pantothenate, urea, caffeine, cystine, and tryptophane brought about an increase in turbidity as compared to the controls. The other compounds shown in table 1 were without effect in this concentration. When 100 micrograms per ml were used, in addition to those compounds just



TABLE 2

*Effect on acid production and glucose utilization by compounds showing growth stimulation for S paratyphi B*

	ACID PRODUCTION		SUGAR UTILIZED	
	2 µg/ml	100 µg/ml	2 µg/ml	100 µg/ml
Nicotinic acid	21*	21	126 0†	260 8
Calcium pantothenate	22	28	184 4	184 4
Urea	18	20	116 0	126 4
Caffeine	21	21	137 6	143 2
Nembutal		19		143 2
Uracil		19		126 4
Creatine		18		172 4
Asparagine		41		190 4
Cystine	37	45	201 2	221 6
Tryptophane	38	41	190 4	211 6
Control	18	18	100 8	100 8

Values obtained are the average of two simultaneous determinations. At least two other determinations were made for each value recorded, with similar results.

\* Figures give ml of N/10 acid per 100 ml medium in 74 hours.

† Figures give mg of glucose utilized per 100 ml medium in 74 hours.

TABLE 3

*Effect of tryptophane, cystine, and asparagine on the sulfonamide inhibition of acid production and sugar utilization by Salmonella paratyphi B*

	ACID PRODUCTION		SUGAR UTILIZATION	
	2 µg/ml	100 µg/ml	2 µg/ml	100 µg/ml
Tryptophane + sulfasuxidine	21*	39	40 8†	172 4
Cystine + sulfasuxidine	39	41	266 4	334 4
Asparagine + sulfasuxidine	0	40		190 4
Control sulfasuxidine	19	19	73 2	73 2
Tryptophane + sulfamerazine	20	40	56 0	271 6
Cystine + sulfamerazine	37	49	276 4	256 4
Asparagine + sulfamerazine	24	49	184 4	307 2
Control sulfamerazine	18	18	61 6	61 6
Tryptophane + sulfaguanidine	32	50	240 0	271 6
Cystine + sulfaguanidine	41	47	271 6	288 8
Asparagine + sulfaguanidine	0	45		292 0
Control sulfaguanidine	21	21	73 2	73 2
Control—no sulfa	32	32	149 2	149 2

Time of fermentation = 74 hours.

\* Figures give ml of acid produced per 100 ml of medium.

† Figures give mg of glucose utilized per 100 ml of medium.

listed, nembutal, uracil, creatine, and asparagine stimulated growth. Thiamine, PABA, and a mixture of thiamine, riboflavin, nicotinic acid, and pyridoxine inhibited growth. Table 1 also shows the effect of these compounds on the

bacteriostatic action of sulfamerazine, sulfaguanidine, and sulfasuxidine. The mixture of the four vitamins listed above enhanced the inhibition of growth exerted by all three sulfonamides. Thiamine and allantoin increased the bacteriostatic action of sulfaguanidine and sulfasuxidine. *Para*-aminobenzoic acid, asparagine, cystine, and tryptophane reversed the inhibiting action of all the sulfonamides.

Table 2 shows the amount of acid produced and the sugar utilized by *S. paratyphi* B in the presence of those compounds which showed stimulation of growth as evidenced by turbidity. A significant increase in acid production was found only for calcium pantothenate, asparagine, cystine, and tryptophane. Sugar utilization for the most part parallels acid production, except that some compounds which showed little evidence of ability to stimulate acid production did show a greater utilization of sugar than did the control. Table 3 shows how acid production and sugar utilization were affected by the sulfonamides and the three amino acids which were most active in stimulating growth. In every case in which 100 micrograms per ml were used, there was a corresponding increase in both acid production and sugar utilization. However, in two cases (2 micrograms of asparagine and sulfamerazine and 2 micrograms of tryptophane and sulfaguanidine) there had been no evidence of a reversal of the action of the sulfonamides, either by turbidity or acid production, but there was an increase in glucose utilization.

#### DISCUSSION

There is considerable difference in response to the same compound by two closely related organisms. *Para*-aminobenzoic acid inhibited growth of both *E. coli* and *S. paratyphi* B, as has been observed for coliform and other organisms many times before. However, several compounds stimulated growth of *S. paratyphi* B which had no effect on *E. coli*. Thiamine inhibited the growth of *S. paratyphi* B but had no such action on *E. coli*, whereas creatine inhibited the growth of *E. coli* but stimulated the growth of *S. paratyphi* B. Strauss, Dingle, and Finland (1941) reported no stimulation of growth of *E. coli* with thiamine, pyridoxine, riboflavin, calcium pantothenate, choline, uric acid, and uracil. We obtained the same results with *E. coli*, although *S. paratyphi* B responded quite differently. Strauss *et al.* reported increased growth for *E. coli* in glucose broth when nicotinic acid was added. We were unable to secure increased growth with *E. coli* when nicotinic acid was used, which is in agreement with the findings of Rantz (1942).

The stimulation of growth by the three amino acids, asparagine, cystine, and tryptophane was the same for both organisms. Lorr and Burrows (1942) reported that tryptophane reduced the lag period for the typhoid bacillus but did not influence the rate of glucose decomposition. They said that tryptophane was not essential but acted as a growth stimulant. Cystine and tryptophane were shown by Burrows (1934) to stimulate the growth of *Clostridium botulinum*. Creatine and asparagine were found by Badger (1944) to increase the growth of type III pneumococcus. We found that there was an increase in glucose utili-

zation and also an increase in acid production by both of the test organisms in the presence of 100 micrograms of each of the three amino acids, asparagine, cystine, and tryptophane. The remainder of the compounds stimulating the growth of *S. paratyphi* B showed no significant increase in acid production, but there was an increase in the glucose utilized. The organisms may have been stimulated to greater growth by using glucose in such a way that acid was not produced.

Allantoin, thiamine, and urea in the concentration of 100 micrograms per ml increased the bacteriostatic action of some of the sulfonamides against one or both of the test organisms. Sulfathiazole, sulfadiazine, and sulfanilamide have been reported to be more effective in the presence of urea (Tsuchiya *et al*, 1942, McClintock and Goodale, 1943, Lee *et al*, 1943). Since allantoin increased the bacteriostatic action with two of the sulfonamides tested against both organisms, it might be worth further investigation to determine, in animals, any curative or protective effects.

Asparagine, tryptophane, and cystine reversed the action of all the sulfonamides for both organisms. This suggests that the sulfonamides are interfering with the synthesis of these amino acids. Since the growth of the organisms is stimulated by small amounts of these acids, they must be unable to synthesize them fast enough for optimal growth. The acid production and sugar utilization in the presence of these amino acids and sulfonamides correspond to the results obtained by observing the turbidity.

#### SUMMARY

The effect of 19 compounds on the growth of *Escherichia coli* and *Salmonella paratyphi* B was determined.

The effect of these compounds on the inhibition of growth of *S. paratyphi* B by sulfamerazine, sulfaguanidine, and sulfasuxidine, and of *E. coli* by sulfamerazine and sulfaguanidine was determined. Allantoin, thiamine, and urea enhanced the bacteriostatic action in some cases.

The acid produced and the glucose utilized were determined in the absence and in the presence of those compounds that stimulated the growth of either organism. Glucose utilization was increased in all cases. A significant increase in acid production was seen only with asparagine, cystine, and tryptophane.

Asparagine, cystine, and tryptophane increased acid production and glucose utilization by *S. paratyphi* B in the presence of sulfamerazine, sulfaguanidine, and sulfasuxidine. This corresponded with the reversal of the bacteriostatic action of the sulfonamides.

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## NOTES

### A NEW SALMONELLA TYPE SALMONELLA MEMPHIS<sup>1</sup>

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In May, 1947, a culture isolated from the stools of a patient with acute diarrhea was received from Mr L S Suter of Kennedy Veterans Administration Hospital, Memphis, Tennessee. The culture was isolated and identified by Miss Kathleen Mason as an unusual *Salmonella* type. The source of the infection was not established.

The organism possessed the usual cultural and biochemical attributes of the *Salmonella* group. Acid and gas were produced from glucose, xylose, rhamnose, arabinose, trehalose, maltose, dulcitol, mannitol, and sorbitol. Lactose, sucrose, raffinose, inositol, and salicin were not attacked. Indole was not produced. The bacterium formed hydrogen sulfide and slowly liquefied gelatin. Citrate, mucate, and *d*-tartrate were utilized, but *l*-tartrate and *α*-tartrate were not fermented.

On serological examination the organism was agglutinated to the titer of *Salmonella* cerro O serum (XVIII) and in absorption tests removed all agglutinins from the serum. The O antigens of *Salmonella memphis* are XVIII.

The H antigens of the culture were diphasic. Phase 1 was agglutinated to the titer of *Salmonella thompson*, phase 1 (k) serum, and was able to remove all agglutinins from the serum. Phase 2 was agglutinated by serums for all the nonspecific phases of the genus. When tested with single factor serums for antigens 2, 3, 5, 6, and 7, it was agglutinated only by 5 serum. In absorption tests *S memphis* was not able to remove completely the agglutinins from serum for phase 2 of *S thompson* (1,5) but left a slight residue that amounted to 1 per cent of the original titer. The antigenic formula of *S memphis* is XVIII k-1,5.

#### SUMMARY

*Salmonella memphis* (XVIII k-1,5) was recovered from the stool of a patient affected with diarrhea. The organism liquefied gelatin slowly.

<sup>1</sup> The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U S Public Health Service.

MODIFICATION OF AN AGAR DIFFUSION METHOD OF ASSAY  
FOR POLYMYXIN

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Polymyxin is an antibiotic elaborated in the fermentation of various media by certain strains of *Bacillus polymyxa* (Stansly *et al* Bull Johns Hopkins Hosp, 81, 43, Benedict and Langlykke J Bact, 54, 24) An agar diffusion method of assay was developed for the antibiotic (Stansly and Schlosser J Bact, 54, 585) From a group of gram-negative organisms tested, *Escherichia coli* (MacLeod strain) was found to be the most satisfactory assay organism However, its use presented certain difficulties since it grows very rapidly, and the antibiotic, which is incorporated in paper disks, diffuses slowly through agar Thus it became necessary to incubate the assay plates at 25 C for 18 hours followed by incubation at 37 C for 6 hours to sharpen the edges of the zones of inhibition

Using essentially the same assay techniques as those of Stansly and Schlosser, we have found that *Brucella bronchiseptica*, NRRL B-140, shows the same order of sensitivity to polymyxin as *Escherichia coli* (MacLeod strain) With *Brucella bronchiseptica*, the inhibition zones have very well-defined edges and are obtained by direct incubation of the assay plates at 37 C for 14 to 16 hours Fermentation liquors also give sharply defined zones For flooding plates the organism is grown in "trypticase soy" broth for 24 hours at 37 C, then 10 ml of undiluted broth is added to each 100 ml of flooding agar, mixed, and 40 ml of the mixture dispensed on each assay plate with an automatic agar dispenser The plates, with covers slightly ajar, are then dried at 37 C in an incubator for 30 minutes They are then removed and held at 20 C until the polymyxin-saturated disks are placed on them Techniques for dilution of fermentation liquors and solid preparations are the same as those employed by Stansly and Schlosser except that the former are first diluted with equal quantities of 0.1 M glycine buffer in sterile 25-by-200-mm test tubes and further dilutions are made with 0.05 M buffer Assay disks may then be saturated directly in these tubes and excess polymyxin removed by touching the disk twice to a dry surface in the upper part of the tube The very slow rate of growth of *Brucella bronchiseptica* during the short 20 C holding period does not require that standard disks be placed on each assay plate All unknown values are calculated from a daily standard curve This curve is obtained by averaging the zones of inhibition on triplicate plates of 256, 128, 64, 32, and 16 polymyxin units per ml, as defined by Stansly and Schlosser (*loc cit*) and plotting them against the log concentration of polymyxin

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# INTERNATIONAL BACTERIOLOGICAL CODE OF NOMENCLATURE

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The following Bacteriological Code of Nomenclature was developed by the Judicial Commission, approved and recommended by the Nomenclature Committee and adopted unanimously at the concluding Plenary Session of the International Association of Microbiologists at the fourth International Congress held in Copenhagen, Denmark, in July, 1947

## Chapter 1

### GENERAL CONSIDERATIONS

1 The progress of bacteriology can be furthered by a precise system of nomenclature which is properly integrated with the systems used by botanists and zoologists and accepted by the majority of bacteriologists in all countries. Bacteriological nomenclature considers bacteria, related organisms, and the viruses. Botanical and zoological codes provide for nomenclature of certain groups such as the yeasts and fungi, protozoa and algae. These are of such significance in the microbiological laboratory that provision is necessary in the bacteriological code for the consideration of special nomenclatural problems in these groups and for co-ordination of findings with zoologists and botanists.

2 The precepts on which this system of bacteriological nomenclature is based are divided into *principles*, *rules*, and *recommendations*.

The *principles* (Chapter 2) form the basis of the rules and recommendations.

The *rules* (Chapter 3) are designed (1) to make effective the principles given in Chapter 2, (2) to put the nomenclature of the past into order, and (3) to provide for that of the future. They are always retroactive; names or forms of nomenclature contrary to a rule (*illegitimate names or forms*) cannot be maintained.

The *recommendations* deal with subsidiary points, their object being to bring about greater uniformity and clearness, especially in future nomenclature; names or forms contrary to a recommendation cannot on that account be rejected, but they are not examples to be followed.

3 *Provisions* for emendation of rules, for special exceptions to rules, and for their interpretation in doubtful cases have been made through the establishment of a Nomenclature Committee for the International Association of Microbiologists and its Judicial Commission (Chapter 4).

<sup>1</sup> Chairman, Judicial Commission

<sup>2</sup> Permanent Secretaries of the Nomenclature Committee for the International Association of Microbiologists

## Chapter 2

## GENERAL PRINCIPLES

*Principle 1* The essential points in nomenclature are (1) to aim at fixity of names, (2) to avoid or to reject the use of forms and names which may cause error or ambiguity or throw science into confusion

Next in importance is the avoidance of all useless creation of names

Other considerations, such as absolute grammatical correctness, regularity or euphony of names, more or less prevailing custom, regard for persons, etc., notwithstanding their undeniable importance, are relatively accessory

(See *Rules 23, 24, 25, 26, 27, Recommendations 27a-1*)

*Principle 2* In the absence of a relevant rule, or where the consequences of rules are doubtful, established custom must be followed. In doubtful cases a résumé in which all pertinent facts are outlined should be submitted to the Judicial Commission for an Opinion

(See *Recommendation 9c, Provision 4*)

*Principle 3* Bacteriological nomenclature and botanical nomenclature are interdependent in the sense that the name of a bacterial group is to be rejected if it is a later homonym of the name of any plant group. Likewise nomenclature of bacteria and protozoa are interdependent, the name of a bacterial group is to be rejected if it is a later homonym of the name of a protozoan group. Bacteriological nomenclature is independent of zoological nomenclature (protozoology excepted), the name of a bacterial group is not to be rejected simply because it is identical with the name of a group in the animal kingdom

(See *Rule 24 (4)*)

*Principle 4* Scientific names of all groups are usually taken from Latin or Greek. When taken from any language other than Latin, or formed in an arbitrary manner, they are treated as if they were Latin. Latin terminations should be used so far as possible for new names

(See *Rules 1-8, 27, 28, Recommendations 5a, 6a, 6b, 6c, 8a, 27a-1*)

*Principle 5* Nomenclature deals with

- (1) The terms which denote the rank of taxonomic groups (such as species, genus, family, order)
- (2) The names which are applied to the individual groups (such as *Bacillus subtilis*, *Streptococcus*, *Spirillaceae*, *Spirochaetales*)

(See *Principle 7, Rules 1-8, Recommendations 6a-c, 8a, 24a*)

*Principle 6* The rules and recommendations of bacteriological nomenclature apply to all bacteria, recent and fossil, with certain distinctly specified exceptions

(See *General Considerations 1, Principle 9, Provisions 2-4*)

*Principle 7* The terms which denote the rank of taxonomic groups are defined as follows

(a) Every individual belongs to a species, every species to a genus, every genus to a family, every family to an order, every order to a class, every class to a division. In some families the rank tribe may be distinguished

(See *Principle 5, Rules 1-8, Recommendations 5a, 6a-c*)

(b) In many species, subspecies or varieties are distinguished, in some cases subdivisions of a species such as strains, groups, serotypes, variants, phases, and others may be recognized. In some genera, subgenera may be distinguished.

(See *Rules 6, 7, Recommendations 6a-c, 8a*)

(c) If a greater number of intermediate categories (ranks) are required, the terms for these subdivisions are made by adding the prefix "sub-" to the terms denoting the ranks. Thus subfamily denotes a rank between a family and a tribe, subtribe a rank between a tribe and a genus, etc. The classification of subordinate categories (ranks) may thus be carried for the bacteria in the following order:

- |                                     |                                     |
|-------------------------------------|-------------------------------------|
| 1 Division ( <i>Divisio</i> )       | 9 Tribe ( <i>Tribus</i> )           |
| 2 Subdivision ( <i>Subdivisio</i> ) | 10 Subtribe ( <i>Subtribus</i> )    |
| 3 Class ( <i>Classis</i> )          | 11 Genus ( <i>Genus</i> )           |
| 4 Subclass ( <i>Subclassis</i> )    | 12 Subgenus ( <i>Subgenus</i> )     |
| 5 Order ( <i>Ordo</i> )             | 13 Species ( <i>Species</i> )       |
| 6 Suborder ( <i>Subordo</i> )       | 14 Subspecies ( <i>Subspecies</i> ) |
| 7 Family ( <i>Familia</i> )         | 15 Variety ( <i>Varietas</i> )      |
| 8 Subfamily ( <i>Subfamilia</i> )   | 16 Individual ( <i>Individuum</i> ) |

(d) The definition of each of these categories (ranks) varies, up to a certain point, according to individual opinion and the state of the science, but their relative order, sanctioned by custom, must not be altered. No classification is admissible which contains such alteration.

*Principle 8* The primary purpose of giving a name to a taxonomic group is not to indicate the characters or the history of the group, but to supply a means of referring to it.

(See *Rule 23*)

*Principle 9* Each group with a given circumscription, position, and rank can bear only one valid name, the earliest that is in accordance with the Rules of Nomenclature. Provisions may be made for certain exceptions.

*Note* In subgenera, genera, and groups of higher rank, the valid name is the earliest name published, provided that this is in conformity with the Rules of Nomenclature.

In species the valid name is the binary and in subspecies the ternary combination containing the earliest epithet published, provided that this combination is in conformity with the Rules of Nomenclature.

(See *Principle 6, Rules 24-26, Provisions 2, 3, 4*)

*Principle 10* Bacteriologists are urged not to change a name (or combination of names) without serious motives, based either on more profound knowledge of facts or on the necessity of giving up a nomenclature that is contrary to the Rules.

*Principle 11* The application of names of taxonomic groups is determined by means of *nomenclatural types*. A nomenclatural type is that constituent element of a group to which the name of the group is permanently attached, whether as an accepted name or as a synonym. The name of a group must be changed if the type bearing that name is excluded.

The type of a generic name is a species, that of the name of a species or sub-

species (variety) is usually an authentic culture, a specimen, or a preparation. In some species, however, the type is a description or a figure given by a previous author. Where permanent preservation of a culture, a specimen, or preparation is impossible, the application of the name of a species or subdivision of a species is determined by means of the original description or figure.

*Note* The nomenclatural type is not necessarily the most typical or representative element of a group, it is merely that element with which the name of the group is permanently associated.

(Examples The type of the name *Bacillus* is the species *Bacillus subtilis*. The type of *Pseudomonas suaveolens* Soppeland is the culture designated and deposited by the author as the type culture in the American Type Culture Collection. The type of *Actinomyces canaliculatus* (Mason) Ford consists of the description and illustration (Jour. Trop. Med. Ther., 1919, 32, 34) as no cultures are available.)

(See Rule 9, Recommendations 9a-d)

*Principle 12* A name of a taxonomic group has no status under the Rules, and no claim to recognition by bacteriologists, unless it is validly published.

(See Rules 10-14, Recommendations 12a-c)

### Chapter 3

#### RULES OF NOMENCLATURE WITH RECOMMENDATIONS

##### Section 1. Naming of Groups of Various Ranks

*Rule 1* Names of divisions, subdivisions, classes, subclasses, orders, suborders, families, subfamilies, tribes, and subtribes are taken either from their chief characters or from a taxonomic unit of the next lower rank.

*Rule 2* The names of all ranks higher than the genus are written in the plural number.

*Rule 3* Names of divisions, subdivisions, classes, and subclasses are words of Greek or Latin origin.

(See Principles 4, 5, 7, Rules 24, 25)

*Rule 4* Names of orders, suborders, families, subfamilies, tribes, and subtribes are also words of Greek or Latin origin or Latinized words, each with a suffix to indicate its taxonomic rank. The suffix for orders is *-ales*, for suborders *-ineae*, for families *-aceae*, for subfamilies *-oideae*, for tribes *-eae*, and for subtribes *-inae*.

(See Principles 4, 5, 7, Rules 22, 24, 25)

*Rule 5* Names of genera and of subgenera are substantives (or adjectives used as substantives) in the singular number and written with an initial capital. These names may be taken from any source whatever and may even be composed in an arbitrary manner. They are treated as Latin substantives. Generic names and subgeneric names are subject to the same rules and recommendations, and, from a nomenclatural standpoint, they are co-ordinate.

Examples *Bacillus*, *Pasteuria*, *Brucella*, *Alcaligenes*, *Fusiformis*  
(See Principles 4, 5, 7)

If a genus is divided into subgenera, one of the subgenera (that which includes the type of the genus) should bear the same name as the genus

Example If the genus *Bacillus* is divided into two or more subgenera, the subgenus which includes the type species *Bacillus subtilis* should bear the subgeneric name *Bacillus* (See Rules 9, 19, 20, 27, 28, Recommendations 9a-c, 19a, 17i )

Recommendation 5a Bacteriologists who are forming new generic or subgeneric names should attend to the following recommendations

- (1) Not to make names very long or difficult to pronounce
- (2) To take names that have an agreeable form readily adaptable to the Latin tongue
- (3) Not to dedicate genera to persons quite unconnected with bacteriology or at least with natural science nor to persons quite unknown
- (4) To avoid adjectives used as nouns
- (5) Not to make names by combining words from different languages (*nomina hybrida*)
- (6) To give a feminine form to all personal generic names, whether they commemorate a man or a woman

(See Principles 4, 7, Rule 27, Recommendations 27a-1 for orthography and gender of generic names )

Rule 6 Names of species are binary combinations consisting of the name of the genus followed by a single specific epithet<sup>3</sup> If an epithet consists of two or more words, these must either be united or joined by hyphens Specific epithets are

- (a) Adjectives, which must agree grammatically with the generic name Examples *Bacillus subtilis*, *Micrococcus aureus*, *Clostridium botulinum*
- (b) Substantives, in the nominative, in apposition with the generic name Examples *Flavobacterium ceramicola*, *Vibrio comma*, *Pseudomonas conjugac*, *Phytomonas holcicola*
- (c) Substantives in the genitive Examples *Phytomonas vascularum*, *Aerobacter cloacae*, *Rhizobium leguminosarum*, *Brucella abortus*, *Acetobacter aceti*, *Salmonella anatis*, *Borrelia lochni*

Within the same genus, no two species names bear the same specific epithet (See Principles 4, 5, 7, Rule 27, and Recommendations 27a-1 for orthography and gender of specific names )

Recommendation 6a When it is desired to indicate the name of a subgenus in connection with the generic name and specific epithet, the name of the subgenus may be placed in parentheses between the two

---

<sup>3</sup> The term "epithet" as here used implies a single descriptive word or a single descriptive phrase

Examples The Latin word *aureus* (golden) is a single descriptive adjective or epithet, and the species name *Micrococcus aureus* would be in correct form The phrase *lac acidum* (sour milk) is a single epithet, and the species name *Streptococcus lactis-acidi* (or *lactisacidi*) (*Streptococcus* of sour milk) is in correct form Care should be used not to regard a sequence of unrelated words as a single epithet The species name *Bacillus aureus lactis* (the golden bacillus of milk) would be an invalid trinomial, there are two specific epithets The name cannot be validated by hyphenating the two words as *Bacillus aureus-lactis*, there are still two unrelated epithets If the two words are combined as in the specific name *Bacillus aurei lactis*, the meaning is completely changed to *Bacillus* of golden milk, the species name is in correct form, but the meaning is nonsensical unless applied to an organism which changes the color of milk to golden

Example *Lactobacillus* (*Thermobacterium*) *caucasicus*

(See *Principles* 4, 5, 7 )

*Recommendation 6b* In forming specific epithets bacteriologists should attend to the following recommendations

- (1) To choose a specific epithet which, in general, gives some indication of the appearance, the characters, the origin, the history, or the properties of the species. If taken from the name of a person, it usually recalls the name of the one who discovered or described it, or was in some way concerned with it

Examples *Micrococcus aureus*, *Clostridium pasteurianum*, *Phylomonas campestris*, *Bacillus viscosus*, *Kurthia zopfii*

- (2) To avoid those which are very long and difficult to pronounce
- (3) To avoid those which express a character common to all or nearly all the species of a genus

Example *Micrococcus sphericus*

- (4) To avoid using the names of little known or very restricted localities, unless the species is quite local
- (5) To avoid, in the same genus, epithets which are very much alike, especially those which differ only in their last letters
- (6) Not to adopt unpublished names found in authors' notes, attributing them to their authors, unless these have approved publication

(See *Principles* 4, 5, 7 )

*Recommendation 6c* Names of men and women and also of countries and localities used as specific epithets may be substantives in the genitive (*welchii*) or adjectives (*pasteurianum*, *japonicum*) It will be well, in the future, to avoid the use of the genitive and the adjectival form of the same epithet to designate two different species of the same genus

(See *Principles* 4, 5, 7 )

*Rule 7* Names of subspecies (varieties) are ternary combinations consisting of the name of the genus followed by the specific and subspecific epithets in order

Example *Escherichia coli* subsp. *communior* (Topley and Wilson) Breed *et al* or *Escherichia coli* var. *communior*, or *Escherichia coli* *communior* This does not justify the name *Bacillus fluorescens liquefaciens*, as this name was originally proposed as a trinomial name for a species, and not for a subspecies or a variety

Epithets of subspecies (varieties) are formed like those of species, when adjectival in form and not used as substantives they agree in gender with the generic name

Neither within the same species nor within the same genus may two subspecies bear the same subspecific epithet

If the species is divided into subspecies, the subspecific epithet of the subspecies containing the type of the species shall be the same as that of the species

Example If *Micrococcus aureus* is divided into two or more subspecies, one (that containing the type) should be designated *Micrococcus aureus* subsp. *aureus*

(See *Principles* 4, 5, 7 )

*Rule 8* Subdivisions of species (other than subspecies (varieties)) are given vernacular names or designated by numerals or letters or, in special cases, as given names in Latin form

(See *Principles* 4, 5, 7 )

**Recommendation Sa** Authors of names of subdivisions of species of bacteria which are not treated as subspecies (varieties) should attend to the following recommendations and definitions

- (1) A *strain* is a pure culture of bacteria made up of the descendants of a single isolation. It is frequently designated by the name of the individual responsible for its isolation, as *Corynebacterium diphtheriae* strain Park-Williams. It may also be designated by the locality or by a number or some similar laboratory distinguishing mark. Strain may also be used to designate cultures of bacteria which correspond to cultivated "varieties" of higher plants in having some special economic significance. Such are frequently names from the laboratory or factory where isolated, as *Acetobacter acetii* strain Carlsberg.
- (2) *Type* is a term which has frequently been used to designate a subdivision of a species, particularly in cases where the differentiating characters are regarded as insufficient to justify the erection of a subspecies or variety. Types are often differentiated on the basis of antigenic characteristics. Type is sometimes used to designate a physiological or morphological variant. In view of the use of the word "type" in a different sense as defined in Principle 11, it is suggested that the terms *serotype* (or *serological type*), *biotype* (or *physiological type*) and *morphotype* (or *morphological type*) may appropriately be substituted for *type* as a designation of a subdivision of a species.
- (3) The term *group* in bacteriology should be used with great care so as to avoid ambiguity. It is employed popularly to designate various organisms with common characteristics (i.e., "Coli-aerogenes Group") and, in a restricted sense, in antigenic analysis for designating species or subgenera (e.g., *Streptococcus* Group A Lancefield), or varieties or subspecies (e.g., *Neisseria intracellularis* Group I Scott). It is suggested that the term *group* be reserved for primary serological divisions and designated by capital letters. Any serological subdivisions within the group should be designated as *types* and distinguished by Arabic numerals (e.g., *Bacterium pseudotuberculosis-rodentium* Group A, Type 1 Schutze)<sup>4</sup>.
- (4) The designation *phase* should be restricted to use for bacteria showing certain alternative immunologic characteristics, and particularly for the "specific phase" or "nonspecific phase" of Andrewes as recorded for the genus *Salmonella*.  
Example *Salmonella enteritidis* specific phase.
- (5) A *form* (*forma*) or *special form* (*forma specialis*) is a subdivision of a species of a parasitic microorganism distinguished primarily by adaptation to a particular host. It is named preferably by giving it the scientific name of the host. This is written preferably in the genitive.  
Example *Rhizobium phaseoli* forma *phaseoli multiflori* or *Rhizobium phaseoli* f. sp. *phaseoli multiflori*.

<sup>4</sup> It may be urged that if specific names are substituted for Group letters at present employed (as strictly speaking they should be) the whole serological concept of the genus is obscured, and it would be difficult to get serologists, working in many of the various fields, to assent to an action that they might consider retrograde. But this procedure has been accepted by the Salmonella Subcommittee in the case of the genus *Salmonella*. However, all workers in this field are familiar with the Kauffmann-White schema which clearly shows the antigenic relations within the genus concept. A somewhat similar procedure might be employed, for example, in the case of the streptococci, i.e., an approved schema drawn up showing the antigenic relationships within the various groups which should be given specific rank with names consistent, so far as possible, with the laws of priority. It is suggested that the Salmonella Subcommittee in this regard has made a sincere effort to reconcile the traditions of the past with the present practical necessity of stressing serological relationships, where they exist.

- (6) A *variant* is an organism showing some variation in some character from the parent culture. Frequently variants result by mutation. If sufficiently distinct and stable, the variant may even be regarded and named as a subspecies or variety. Example of variant: the progeny of a color sector in a pigmented colony, or the progeny of secondary colonies arising as lactose-fermenting mutants in colonies of glucose-fermenting bacteria.

Example *Shigella sonnei* lactose-positive variant

- (7) A *stage* or *state* is the name given to the Rough, Smooth, Mucoid, and similar variants which arise from colonies of many species of bacteria. These are regarded as alternating stages which are generally reversible and indeed by some authors as part of a pleomorphic life cycle. They may be designated by some vernacular descriptive name.

Example *Bacillus subtilis* Rough stage

(See *Principles* 4, 5, 7b)

4

## Section 2 Designation of Nomenclatural Types

**Rule 9** For each valid name of each taxonomic group there should be designated a type, that is for each species or subspecies a type culture, specimen, or description, for each genus a type species (genotype).

(See *Principle* 11, *Rule* 5)

**Recommendation 9a** When publishing names of new taxonomic groups, authors should indicate carefully the subdivision which is the type of the new name. The type species (genotype) in a genus, the type subspecies or variety in a species in which these subdivisions are recognized, the type specimen, preparation, or description in a species. This type determines the application of the name in the event of the taxonomic group being subsequently divided. When describing new species, varieties, or forms of parasitic bacteria, the host of the type should be indicated.

(See *Principle* 11, *Rule* 5)

**Recommendation 9b** When revising a genus for which no genotype has been designated, an author should state which species he accepts as the nomenclatural type.

(See *Principle* 11, *Rule* 5)

**Recommendation 9c** In selecting a nomenclatural type (genotype) for a genus of bacteria, bacteriologists should, when possible, choose a species that will fix the generic name as it is now commonly applied.

(See *Principles* 2, 11, *Rule* 5)

**Recommendation 9d** The utmost importance should be given to the preservation of the original ("type") material on which the description of the new group is based. The original account should state where this material is to be found. When a new species or subspecies of bacterium is described, if the organism is one which may be maintained in pure culture, an authentic culture labeled as "type" should be deposited with one of the recognized national or international type culture collections. The national or international type culture depositories recognized are designated by the action of the International Committee on Nomenclature. These recognized in 1939 are the National Collection of Type Cultures of Microorganisms maintained at the Lister Institute, London, England, and the American Type Culture Collection, Washington, D. C. Inasmuch as the type of a bacterial species is frequently the published description and drawings, these should be as complete as possible.

**Note** It should be borne in mind that morphological, biochemical and antigenic changes, and also loss of virulence, may take place as the result of repeated subculture in the collection. This can to some extent be obviated by drying cultures in high vacuum under optimal conditions and storing them for future reference.

(See *Principle* 11)



### Section 3. Publication of Names

*Rule 10* Legitimate bacteriological nomenclature begins with Linnaeus' *Species Plantarum*, ed 1, 1753<sup>5</sup>

(See *Principle 12* )

*Rule 11* Publication is effected, under these Rules, by sale or distribution of printed matter to the general public or to bacteriological institutions. No other kind of publication is accepted as *effective* (*effective publication*), communication of new names at a public meeting, or the placing of names in collections, does not constitute effective publication.

Where reprints or separates from periodicals or other works are placed on sale or issued in advance, the date on the separate is accepted as the date of effective publication.

The date of acceptance of an article for publication as given in a publication does not indicate the effective date of publication and has no significance in determination of priority of publication of names.

(See *Principle 12*, *Rule 12* )

*Rule 12* A name of a taxonomic group is not validly published unless it is both (1) effectively published (See *Rule 11*), and (2) accompanied by a description of the group or by a reference to a previously and effectively published description of it.

The words "valid" or "validly published" as used in these Rules mean "with standing in nomenclature," and the words "invalid" or "not validly published" mean "without standing in nomenclature."

Mention of a name on a label on a culture or preparation of bacteria in a collection without printed or autographed description does not constitute valid publication of that name.

A name of a taxonomic group is validly published only if it has been definitely accepted by the author who published it. A name proposed provisionally (*nomen provisorium*) in anticipation of the eventual acceptance of the group, or of the circumscription, position, or rank given to a group, or mentioned only incidentally is not validly published.

*Example* Beijerinck (*Arch neerl d sc exactes*, 1903, Sec 2, 8, 217) mentioned in a footnote to his article describing and naming the genus *Azotobacter* that *Parachromatium* might be a suitable name. It was never formally proposed or adopted, and has no standing in nomenclature.

A name of a taxonomic group is not validly published when it is merely cited as a synonym.

*Example* Trevisan (*Rendiconti Real Ist Lombard d Sci e Lett*, Ser 2, 1879, 12, 144) cited *Malleomyces equestris* Hallier as a synonym of *Micrococcus equestris*, which he regarded as the causal organism of glanders. Inasmuch as all of Hallier's species were based upon mixed cultures and his names invalid, this incidental citation as synonym by Trevisan does not validate the name. *Malleomyces* must date as a generic name from its proposal by Pribram in 1933 (*Klassifikation des Schizomyceten*, p 93).

<sup>5</sup> Fixed by action of the First International Congress of Microbiology in Plenary Session, Paris, 1930 (*Proceedings*, Part 2, p 527).

A group is not characterized, and the publication of its name is not validated merely by mention of the subordinate groups included in it thus, the publication of the name of an order is not validated by mention of the included families, that of a family is not validated by mention of the included genera, that of a genus is not validated by mention of the included species

The date of a name or of an epithet is that of its valid publication For purposes of priority, however, only legitimate names and epithets published in legitimate combinations are taken into consideration In the absence of proof to the contrary, the date given in the work containing the name or epithet must be regarded as correct

(See *Principle 12*, *Rule 27*, *Note 1* )

**Example** *Chondromyces crocatus* Berkeley and Curtis 1857 (in Berkeley, *Introduction to Cryptogamic Botany*, p 313) is a name appended to an illustration without description The description was published later (Berkeley, *Grevillea*, 1874, 3, 64) and valid publication was of the later date

**Recommendation 12a** When publishing names of new groups of bacteria in works written in a language unfamiliar to the majority of workers in bacteriology, it is recommended that the authors publish simultaneously the diagnoses in a more familiar language

(See *Principle 12* )

**Recommendation 12b** Authors should indicate precisely the date of their works In the case of a work appearing in parts, the last published sheet of the volume should indicate the precise dates on which the different fascicles or parts of the volume were published as well as the number of pages in each

(See *Principle 12* )

**Recommendation 12c** When works are published in periodicals, the author should require the publisher to indicate on the separates or reprints the date (year and month, if possible the day) of publication and also the title of the periodical from which the work is extracted Separates or reprints should always bear pagination of the periodical of which they form a part, if desired, they may also bear a special pagination

(See *Principle 12* )

**Rule 13** A name of a genus is not validly published unless it is accompanied (1) by a description of the genus, or (2) by the citation of a previously and effectively published description of the genus under another name, or (3) by a reference to a previously and effectively published description of the genus as a subgenus, or other subdivision of a genus

The name of a monotypic new genus based on a new species is validated by the provision of a combined generic and specific description

(See *Principle 12* )

**Examples of validly published generic names** *Bacillus* Cohn 1872, *Pasteurella* Trevisan 1885, *Sarcina* Goodsir 1842, *Polyangium* Link 1809

**Rule 14** The name of a species or a subspecies (variety) is not validly published unless it is accompanied (1) by a description of the group, or (2) by the citation of a previously and effectively published description of the group under another name

(See *Principle 12* )

**Example of validly published name of species** *Bacillus subtilis* Cohn 1872

## Section 4 Citation of Authors and Names

**Rule 15** For the indication of the name (unitary, binary, or ternary) of a group to be accurate and complete, and in order that the date may be readily verified, it is necessary to cite the author who first published the name in question

Examples *Plocamobacteriales* Pribram (or Pribram 1933), *Proteus* Hauser (or Hauser 1885), *Serratia marcescens* Bizio (or Bizio 1823)

An alteration of the diagnostic characters or of the circumscription of a group without exclusion of the type does not warrant the citation of an author other than the one who first published the name. When the changes have been considerable, an indication of their nature and of the author responsible for the change is added, as, *em* (*emendavit*) or *mutatis charact*, or *pro parte*, or *excl gen*, *excl sp*, *excl var*, or some other abridged indication

Example *Bacillus* Cohn *em* Migula

When a name of a taxonomic group has been proposed but not published by one author, and is subsequently validly published and ascribed to him (or her) by another author who supplied the description, the name of the latter author must be appended to the citation with the connecting word *ex*. If it is desirable or necessary to abbreviate such a citation, the name of the publishing author, being the more important, must be retained

Example *Salmonella dar-es-salaam* Schütze *ex* Brown, Duncan and Henry

When a name and description by one author are published by another author, the word *apud* is used to connect the names of the two authors, except where the name of the second author forms part of the title of a book or periodical, in which case the connecting word *in* is used instead

**Rule 16** When a genus, a subgenus, a species, or a subspecies (variety) is altered in rank but retains its name or epithet, the original author must be cited in parentheses, followed by the name of the author who effected the alteration. The same holds when a subgenus, a species, or a subspecies (variety) is transferred to another genus or species with or without alteration of rank

Example *Spirochaete pallida* Schaudinn and Hoffman becomes *Treponema pallidum* (Schaudinn and Hoffman) Schaudinn

**Recommendation 16a** When citing a name published as a synonym, the words "as synonym" or "pro synon" should be added to the citation

When an author publishes as a synonym a manuscript name of another author, the word *ex* should be used to connect the names of the two authors

**Recommendation 16b** When citing in synonymy a name invalidated by an earlier homonym, the citation should be followed by the name of the author of the earlier homonym preceded by the word "non," preferably with the date of publication added. In some instances it will be advisable to cite also any later homonym or homonyms

Example *Myzococcus* Gonnerman 1907 *non* Thaxter 1892

## Section 5 Changes in Names as a Result of Segregation or Union of Groups or Change in Rank of Groups

**Rule 17** An alteration of the diagnostic characters, or of the circumscription of a group, does not warrant a change in its name except insofar as this may be necessitated (1) by transference of the group or (2) by a change of its rank

When a genus is divided into two or more genera, the generic name must be retained for one of them, or (if it has not been retained) must be re-established. When a particular species was originally designated as the type, the generic name must be retained for the genus including that species. When no type was designated, a type must be chosen.

Example Donker (1926) divided the genus *Bacillus* into *Bacillus* and *Aërobacillus*, retaining *Bacillus* for the genus containing the type species *Bacillus subtilis*.

The same rule is applied when a subgenus is divided into two or more subgenera.

**Rule 18** When a species is divided into two or more species, the specific epithet must be retained for one of them, or (if it has not been retained) must be re-established. When a particular specimen was originally designated as the type, the specific epithet must be retained for the species including that specimen. When no type was designated, a type must be chosen according to the regulations given.

The same rule applies to subspecies (varieties), for example, to a subspecies (variety) divided into two or more subspecies (varieties).

Example When *Rhizobium leguminosarum* Frank was divided into several species, all symbiotic on the roots of leguminous plants, the name *R. leguminosarum* was correctly retained for one of them by Fred.

When a species is transferred to another genus (or placed under another generic name for the same genus), without change of rank, the specific epithet must be retained or (if it has not been retained) must be re-established unless one of the following obstacles exists: (1) the resulting binary name is a later homonym or tautonym or (2) there is available an earlier validly published specific epithet.

When the specific epithet, on transference to another generic name, has been applied erroneously in its new position to a different species, the new combination must be retained for the organism on which the epithet was originally based.

**Rule 19** When two or more groups of the same rank are united, the oldest legitimate name or (in species and their subdivisions) the oldest legitimate epithet is retained. If the names or epithets are of the same date, the author who unites the group has the right of choosing one of them. The author who first adopts one of them, definitely treating another as a synonym or referring it to a subordinate group, must be followed.

(See Rule 5)

*Recommendation 19a* Authors who have to choose between two generic names should note the following recommendations

- (1) Of two names of the same date to prefer the one which was first accompanied by the description of a species
- (2) Of two names of the same date, both accompanied by descriptions of species, to prefer the one which, when the author made his choice, included the larger number of species
- (3) In cases of equality from these various points of view to prefer the more correct and appropriate name

(See *Rule 5* )

*Rule 20* When several genera are united as subgenera under one generic name, the subgenus including the type of the generic name used must bear that name unaltered

(See *Rule 5* )

*Rule 21* When several species are united as subspecies or varieties under one specific name, the subdivision which included the type of the specific epithet used must be designated by the same epithet unaltered

*Rule 22* (1) When a subtribe becomes a tribe, when a tribe becomes a subfamily, when a subfamily becomes a family, etc , or when the inverse changes occur, the stem of the name should not be altered but only the termination (-*inae*, -*ae*, -*oideae*, -*aceae*, -*ineae*, -*ales*, etc )

(2) When a subgenus becomes a genus, or the inverse changes occur, the original name should be retained

(3) When a subdivision of a species becomes a species, or the inverse change occurs, the original epithet should be retained unless the resulting combination is rejected under Section 6

(See *Rules 3, 4* )

## Section 6 Rejection and Replacement of Names

*Rule 23* A name or epithet must not be rejected, changed, or modified merely because it is badly chosen or disagreeable, or because another is preferable or better known

(See *Principles 1, 8, 10* )

*Rule 24* A name must be rejected if it is illegitimate, i e , if it is contrary to a rule The publication of an epithet in an illegitimate combination must not be taken into consideration for purposes of priority

(See *Principle 1, Rules 1-4* )

A name of a taxonomic group is illegitimate in the following cases

- (1) If it was nomenclaturally superfluous when published, i e , if the group to which it was applied, as circumscribed by its author, included the type of a name which the author ought to have adopted under one or more of the Rules

Example *Dicrobactrum* Enderlein 1917 was superfluous because of the previous publication of *Serratia* Bizio 1823

- (2) If it is a binary or ternary name published in contravention of *Principle 9* and *Rules 17-23*, i.e., if its author did not adopt the earliest legitimate epithet available for the group with its particular circumscription, position, and rank
- (3) If its specific epithet must be rejected under *Rule 25*
- (4) If it is a later homonym of a genus of bacteria, of a genus of plants, or of a genus of protozoa, that is, if it duplicates a name previously and validly published for a group of the same rank based on a different type. Even if the earlier homonym is illegitimate, or is generally treated as a synonym on taxonomic grounds, the later homonym must be rejected. When an author simultaneously publishes the same new name for more than one group, the first author who adopts one of them, or substitutes another name for one of them, must be followed

*Recommendation 24a* Authors should avoid introducing into bacteriology as generic names such names as are in use in zoology

(See *Principle 5*)

*Note* Mere orthographic variants of the same name are treated as homonyms when they are based on different types

(See *Rule 28*)

- (5) If, owing to a segregation, it is used with different meanings, and so becomes a permanent source of confusion or error. A list of names to be abandoned for this reason will be included under *nomina rejicienda*  
(See *Principle 3, Provision 3*)
- (6) If its application is uncertain (*nomen dubium*). A list of names to be abandoned for this reason will be included under *nomina rejicienda*  
(See *Provision 3*)
- (7) If the characterization of the group was based upon an impure or mixed culture. A list of names to be abandoned for this reason (*nomina confusa*) will be included under *nomina rejicienda*  
(See *Provision 3*)

*Examples* The characters of the genus *Malleomyces* Hallier 1870 were derived from various fungi and bacteria erroneously supposed to be growth forms of a single organism. The name *Salmonella tokio* Aoki was based upon a mixed culture

- (8) If it was based upon an abnormality

*Example* An eroded colony of *Shigella dysenteriae* due to bacteriophage action would be such an abnormality

*Rule 25* Specific epithets are illegitimate in the following special cases and must be rejected

- (1) When they are merely words not intended as names
- (2) When they are merely ordinal adjectives being used for enumeration
- (3) When they exactly repeat the generic name (Tautonym)  
(See *Principles 1, 9, Rules 1, 2*)

*Rule 26* The name or epithet to be rejected according to *Rules 23-25* is replaced by the oldest legitimate name, or (in a combination) by the oldest

legitimate epithet which will be, in the new position, in accordance with the Rules. If none exists, a new name or epithet must be chosen. Where a new epithet is required, an author may, if he wishes, adopt an epithet previously given to the group in an illegitimate combination, if there is no obstacle to its employment in the new position or sense.

(See *Principles* 1, 9)

### Section 7 Orthography and Gender of Names

**Rule 27** The original spelling of a name or epithet must be retained, except in the case of a typographical error, or of a clearly unintentional orthographic error. When the difference between two generic names lies in the termination, these names must be regarded as distinct, even though differing by one letter only. This does not apply to mere orthographic variants of the same name.

**Example** *Streptococcus erysipelatos* and *S. erysipelatis* are mere orthographic variants of the same name. *Erysipelatos* is the strict transliteration of the Greek genitive, *erysipelatis* is the form of the more usual and preferable transliteration into Latin form.

(See *Principles* 1, 4, 9, Rules 5, 6 (c), *Recommendation* 5a.)

**Note 1** The words "original spelling" in this Article mean the spelling employed when the name was validly published.

(See *Rules* 1, 2)

**Note 2** The use of a wrong connecting vowel or vowels (or the omission of a connecting vowel) in a specific epithet, or in the name of a genus is treated as an unintentional orthographic error which may be corrected.

**Note 3** In deciding whether two or more slightly different names should be treated as distinct or as orthographic variants, the essential consideration is whether they may be confused with one another or not. If there is a serious risk of confusion they should be treated as orthographic variants. Doubtful cases should be referred to the Judicial Commission for an Opinion.

**Note 4** Specific and other epithets and names of Greek origin differing merely by having Greek and Latin terminations respectively are orthographic variants. Epithets bearing the same meaning and differing only slightly in form are considered as orthographic variants. The genitive and adjectival forms of a personal name are, however, treated as different epithets.

**Example** *Hormodendron* and *Hormodendrum*, the strict transliteration of the Greek neuter ending is *-on*. The usual and preferable transliteration into the Latin is *-um*.

The liberty of correcting a name must be used with reserve, especially if the change affects the first syllable, and above all the first letter of the name.

**Recommendation 27a** When a new name is derived from a Greek word containing the *spiritus asper* (rough breathing), this should be transcribed as the letter *h*.

(See *Principles* 1, 4, *Recommendation* 5a, *Rule* 6 (c).)

**Recommendation 27b** For scientific names it is advisable to use another font than that used for the remainder of the text, or to space the letters, or to use some similar device appropriate to the text.

**Example** "The disease anthrax is caused by *Bacillus anthracis* Koch." Typewritten scientific names should be underlined.

(See *Principles* 1, 4, *Recommendation* 5a, *Rule* 6 (c).)

**Recommendation 27c** When a new name for a genus or subgenus is taken from the name of a person, it should be formed in the following manner

- (1) When the name of the person ends in a vowel the letter *a* is added (thus, *Gaffky*, after Gaffky, *Noguchia* after Noguchi, *Serratia* after Serratii), except when the name already ends in *a*, when *ea* is added (e g , *Collaea* after Colla)
- (2) When the name of a person ends in a consonant the letters *ia* are added (e g , *Escherichia* after Escherich, *Erwinia* after Erwin F Smith, *Pasteuria* after Pasteur), except when the name ends in *er*, when *a* is added (e g , *Kernera* after Kerner)
- (3) Names may be formed by use of a prefix or a suffix, or modified by anagram or abbreviation. In these cases they count as different words from the original name.

In many cases the names of bacterial genera are formed from the names of persons by the addition of a diminutive ending. The most common modern Latin convention is to add one of the endings *-ellus*, *a*, *um*, preferably *-ella* to conform to *Recommendation 5a*. In some few cases one of the endings *-illus*, *a*, *um* has been added.

(See *Principles 1, 4, Recommendation 5a, Rule 6 (c)*)

- (4) The syllables which are not modified by these endings retain their original spelling, even with the consonants *k* and *w* or with the groupings of vowels which were not used in classical Latin. Letters foreign to botanical Latin should be transcribed and diacritic signs suppressed. The Germanic *ā*, *ō*, *ū* become *ae*, *oe*, *ue*, the French *ê*, *è*, and *é* become generally *e*. In works in which diphthongs are not represented by special type, the diaeresis sign should be used where required, e g , *Aërobacillus* not *Aerobacillus*.

*Recommendation 27d* A new specific or subspecific (varietal) epithet taken from the name of a man may assume either a substantival or an adjectival form. The syllables which are not modified by these endings retain their original spelling, even with the consonants *k* or *w* or with the groupings of vowels which were not used in classical Latin. Letters foreign to botanical Latin should be transcribed and diacritic signs suppressed. The Germanic *ā*, *ō*, *ū* become *ae*, *oe*, *ue*. The French *ê*, *è*, *é* become generally *e*.

When the epithet is a substantive, it is formed in the following manner:

- (1) When the name of the person ends in a vowel, the letter *i* is added (thus, *sonnei* from Sonne) except when the name ends in *a*, when *e* is added (thus, *balansae* from Balansa).
- (2) When the name ends in a consonant, the letters *ui* are added (thus, *welchui* from Welch) except when the name ends in *er*, when *i* is given (thus, *barkeri* from Barker).

When the epithet is an adjective, it is formed by the addition of an appropriate ending (thus, *pasteurianus*, *a*, *um* from Pasteur).

(See *Principles 1, 4, Recommendation 5a, Rule 6 (c)*)

*Recommendation 27e* The same provisions apply to epithets formed from the names of women. When these have a substantival form they are given a feminine termination (Thus, *Cytophaga l rzemieniowski ae*).

(See *Principles 1, 4, Recommendation 5a, Rule 6 (c)*)

*Recommendation 27f* New specific (or other) epithets should be written in conformity with the original spelling of the words from which they are derived and in accordance with the rules of Latin and latinization.

Examples *silvestris* (not *sylvestris*), *sinensis* (not *chinensis*)

(See *Principles 1, 4, Recommendation 5a, Rule 6 (c)*)

*Recommendation 27g* Specific epithets, even those derived from names of persons, should not be capitalized.

(See *Principles 1, 4, Recommendation 5a, Rule 6 (c)*)

*Recommendation 27h* In the formation of names or epithets composed of two or several roots taken from Latin or Greek, the vowel placed between the two roots becomes a connecting vowel, in Latin usually *i*, in Greek usually *o*. When the second root begins with a vowel and euphony requires, the connecting vowel should be eliminated (e g , *lepidantha*). The connecting vowels *ae* should be retained only where this is required for etymological reasons (e g , *caricaeformis* from *Carica*, in order to avoid confusion with *cariciformis*).



from Carex) In certain compounds of Greek words, no connecting vowel is required, e g , *brachycarpus* and *glycyphyllus*

(See *Principles* 1, 4, *Recommendation* 5a, *Rule* 6 (c) )

*Recommendation* 27i Authors should give the etymology of new generic names, and also of new epithets when the meaning of these is not obvious

(See *Principles* 1, 4, *Rule* 5, *Recommendation* 5a, *Rule* 6 (c) )

*Rule* 28 The gender of generic names is governed by the following regulations

- (1) A Greek or Latin word adopted as a generic name retains its classical gender In cases where the classical gender varies the author has the right of choice between the alternative genders In doubtful cases general usage should be followed
- (2) Generic names which are modern compounds formed from two or more Greek or Latin words take the gender of the last If the ending is altered, however, the gender will follow it

Example *Spirochaete* is feminine because the Greek noun *chaete* (χαίτη) is feminine However, if a name *Spirochaetum* were proposed it would be neuter

- (3) Arbitrarily formed generic names or vernacular names used as generic names take the gender assigned to them by their authors Where the original author has failed to indicate the gender, the next subsequent author has the right of choice

(See *Principle* 4, *Rule* 5 )

## Chapter 4

### PROVISIONS FOR EXCEPTIONS TO THE RULES AND FOR THE INTERPRETATION AND MODIFICATION OF RULES

*Provision* 1 *Modification and amendment of Rules* These Rules can be amended only by action of a plenary session of an International Congress for Microbiology convened by the International Association of Microbiologists

*Provision* 2 Lists of *nomina conservanda* To avoid disadvantageous changes in the nomenclature of the genera by the strict application of the Rules of Nomenclature, the Rules provide for a list of names which must be retained as exceptions (*nomina conservanda*)

*Note* 1 This list of conserved names will remain permanently open for additions Any proposal of an additional name must be accompanied by a detailed statement of the case for and against its conservation Such proposals must be submitted to the Judicial Commission (see *Provision* 4) for study and appropriate action

*Note* 2 When a name proposed for conservation has been provisionally approved by the Judicial Commission, bacteriologists are authorized to retain it pending the decision of the next International Congress for Microbiology

*Note* 3 A conserved name is conserved against all other names for the group, whether these are cited in the corresponding list of rejected names or not, so long as the group concerned is not united with another group bearing a legitimate name In the event of union or reunion with another group, the earlier of the two competing names is adopted in accordance with *Rules* 19, 20, and 21

*Note 4* A conserved name is conserved against all earlier homonyms

*Example* The generic name *Bacillus* Cohn with the type species *B. subtilis* Cohn *em* Prazmowski is conserved by recommendation of the Nomenclature Committee and the action of the Second International Congress for Microbiology

(See *Principles* 6, 9, *Rule* 24 (5), (6), (7) )

*Provision 3* Lists of *nomina rejicienda* To avoid unnecessary confusion in the nomenclature of bacteria by the strict application of the rules of nomenclature, the Rules provide a list of names (*nomina rejicienda*) which are not to be used, i e, are to be permanently rejected This list includes names which, owing to segregation, are used with different meanings and have become a permanent source of confusion or error (*nomina ambigua*), names where application is uncertain (*nomina dubia*), and names applied to a group made up of two or more discordant elements, especially if these elements were erroneously supposed to form part of the same individual (*nomina confusa*)

(See *Principles* 6, 9, *Rule* 24 (5), (6), (7) )

*Note 1* This list of rejected names will remain permanently open for additions Any proposal of an additional name must be accompanied by a detailed statement of the case for and against its rejection Such proposals must be submitted to the Judicial Commission of the Nomenclature Committee for study and appropriate action When a name proposed for rejection has been provisionally rejected by the Judicial Commission, bacteriologists are authorized to reject it pending the decision of the next International Congress for Microbiology

*Note 2* A rejected name may not be later introduced into bacteriological literature, except that *nomina dubia* may be removed from the list upon submission of evidence of correct status and by action by the Judicial Commission on Nomenclature

*Provision 4* *Authorization of a Nomenclature Committee* A permanent Nomenclature Committee has been established by the International Association of Microbiologists in Congress This Nomenclature Committee is so constituted that wherever practicable each nation is represented by at least one member, and no nation by more than five Recommendations for nomination for membership on this Nomenclature Committee may be made by any society of microbiologists or by members of any International Congress Recommendations for nominations should be made to one of the Permanent Secretaries who will present them to the Nomenclature Committee for consideration at its next meeting Appointments to membership on the Nomenclature Committee are made by nomination by the Nomenclature Committee and election by the next following Plenary Session of an International Congress for Microbiology The International Congress elects two Permanent Secretaries, one primarily to represent medical bacteriology and one to represent nonmedical bacteriology The Nomenclature Committee shall elect such other officers as may be desired A complete list of all members of the Nomenclature Committee shall be published in the *Proceedings* of each triennial meeting of the International Congress for Microbiology

The Nomenclature Committee selects from its membership a Judicial Commission consisting of twelve members, exclusive of members *ex officio*, and designates a Chairman from the membership of the Commission The two Perma

nent Secretaries of the Nomenclature Committee are members *ex officio* of the Judicial Commission. The commissioners serve in three classes of four commissioners each for nine years, so that one class of four commissioners retires at each International Congress. In the event of failure of the International Congress to meet triennially, the term of office of each class will automatically be extended by the number of years greater than three elapsing between successive Congresses. In case of resignation or death of any commissioner, his place shall be filled for the unexpired term by the Nomenclature Committee at its next meeting.

A The Nomenclature Committee has the following functions

- (1) To consider and pass upon all recommendations relating to the formulation or modification of Rules of Nomenclature, particularly such rules as relate to bacteria, but also pertaining to nomenclature of other groups when desirable. The Committee will recommend such action as may be appropriate to the next Plenary Session of an International Congress for Microbiology.
- (2) To consider all Opinions rendered by the Judicial Commission. Such Opinions become final if not rejected at the meeting of the International Committee next following the date on which the Opinion was issued.
- (3) To designate official Type Culture Collections.
- (4) To receive and act upon all reports and recommendations received from the Judicial Commission or other committees relating to problems of nomenclature or taxonomy.
- (5) To hold at least one meeting triennially in connection with the meeting of the International Congress for Microbiology.
- (6) To report to the final Plenary Session of each Congress a record of its actions, and to recommend for approval such actions and nominations as require the approval of the Congress.
- (7) To co-operate with other Committees, particularly those of the International Botanical and Zoological Congresses, to consider common problems of nomenclature.

(See *General Considerations* 1.)

B The Judicial Commission of the Nomenclature Committee has the following functions

- (1) To issue formal "*Opinions*" when asked to interpret rules of nomenclature in cases in which the application of a rule is doubtful.
- (2) To prepare formal "*Opinions*" relative to the status of names which have been proposed, placing such names when deemed necessary in special lists, such as lists of *nomina conservanda*, *nomina rejicienda*, etc.
- (3) To develop recommendations for emendations of the International Rules for Bacteriological Nomenclature, for presentation to the Nomenclature Committee.
- (4) To prepare formal "*Opinions*" relative to types, particularly types of species and genera, and to develop a list of bacterial genera which have been proposed with the type species of each.

- (5) To prepare and publish lists of names of genera which have been proposed for bacteria, for protozoa, or for other groups in which microbiologists are interested in order to assist authors of new names in avoiding invalid homonyms
- (6) To develop a list of publications in microbiology whose names of organisms shall have no standing in bacteriology in determination of priority
- (7) To edit and publish the International Rules of Bacteriological Nomenclature, Opinions, Lists of *Nomina Conservanda*, *Nomina Rejcienda*, Type Species, etc
- (8) To report to the Nomenclature Committee at its triennial meetings all Recommendations, Transactions, and Opinions
- (9) To report to the International Committee at its triennial meetings the names of all Commissioners whose terms of service expire, likewise a list of all vacancies caused by resignation or death
- (10) To prepare "*Opinions*" when requested relative to the nomenclatural status of microorganisms studied by microbiological techniques, but not classed with the bacteria or viruses, for example the yeasts, molds, and protozoa. However, such "*Opinions*" shall not be issued until confirmed by the commission charged with the interpretation of the appropriate code of nomenclature (Botanical or Zoological)

*Recommendation 4* Whenever, in the opinion of any microbiologist an interpretation of any rule or recommendation of nomenclature is desirable because the correct application of such a rule or recommendation is doubtful, or the stability of nomenclature could be increased by the conservation or by the rejection of some name which is a source of confusion or error, it is recommended that he prepare a résumé outlining the problem, citing pertinent references, and indicating reasons for and against specific interpretations. This résumé should be submitted to the Chairman of the Judicial Commission, if desired, through one of the Permanent Secretaries. An Opinion will be formulated, which may not be issued until it has been approved by at least eight members of the Commission.

(See *Principles* 2, 6 )

# DEMONSTRATION OF PHOSPHATASES AND LIPASE IN BACTERIA AND TRUE FUNGI BY STAINING METHODS AND THE EFFECT OF PENICILLIN ON PHOSPHATASE ACTIVITY<sup>1</sup>

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A considerable amount of work is being done on the histochemical localization of some enzymes in the tissues of higher animals and plants. However, although bacteria have been frequently studied for a few isolated substances such as glycogen, lipids, nucleic acids, and protein, no determined effort has been made to analyze these lower forms of life for their various enzyme systems by histochemical methods. The localization of enzymes and chemical constituents in bacteria offers possibilities of determining further details of their internal anatomy, and may help to shed light on the problem of the bacterial nucleus. In addition, such studies may aid in classifying microorganisms as well as in giving a better understanding of their physiology.

This communication describes the results of applying the methods of Gomori (1946 *a, b*) for the localization of acid and alkaline phosphatases and lipase to bacteria and true fungi. The effect of penicillin, in concentrations sufficient to halt growth, on the acid and alkaline phosphatase stains of certain bacteria was also studied, and the findings were checked with the quantitative effect of this antibiotic on the phosphatase activities in the culture suspensions of the same organisms.

Roche *et al.* (1946) reported that the exposure of dialyzed intestinal tissue extracts to divalent cations such as those of magnesium and manganese in combination with alanine resulted in an activation of the alkaline phosphatase present. Accordingly, in this investigation magnesium and manganous ions along with alanine in each case were added separately to two lots of agar media in order to determine the effect of these agents on the staining, not only of the alkaline phosphatase, but also of the acid phosphatase and lipase in the bacteria grown on these media.

## EXPERIMENTAL PROCEDURES

Bacterial smears were prepared in the usual manner by allowing a suspension of the organisms in distilled water to dry on microscope slides. The slides were not heated. After drying they were placed in a Coplin jar full of acetone for 10 minutes, to be fixed. The acetone was rinsed from the slides with distilled water, after which they were placed in a 0.1 M citrate buffer, pH 4.7, to remove

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naturally occurring inorganic phosphates and other substances that might give a positive test. Thus any staining that was obtained was due to enzyme activity alone. The wash interval may vary, but 0.5 of an hour was found to be sufficient. The slides were then removed, washed thoroughly, and placed in the substrate solution to be incubated overnight. During the incubation period the enzyme, if present, hydrolyzes the substrate, releasing an anion that is immediately precipitated, *in situ*, by cations in the substrate media. The precipitate is then converted to a compound that can be visualized in the microscope.

The principle of the Gomori method is as follows. The alkaline phosphatase activity is visualized by precipitation of the phosphate, which is liberated from the substrate, as the calcium salt; this compound is converted to cobalt phosphate, and finally transformed to cobalt sulfide, which is black and can be easily seen. In the case of acid phosphatase, lead is used in place of calcium because calcium phosphate is soluble at the lower pH values used. Lipase is visualized by the formation of a precipitate of the calcium salt of the high molecular weight fatty acids that are set free by the enzyme action. This precipitate is then converted to lead sulfide in the same manner as for phosphatase.

### *Alkaline Phosphatase*

#### *Reagents*

(1) *Citrate buffer*. Add 2 volumes of 0.1 M hydrochloric acid to 8 volumes of citrate solution (21 g of citric acid plus 200 ml of 1 M sodium hydroxide made up to 1 liter with distilled water).

(2) *Substrate*. Combine 25 ml of 2 per cent sodium glycerophosphate,<sup>2</sup> 25 ml of 2 per cent sodium barbital, 50 ml of distilled water, 5 ml of 2 per cent calcium chloride, and 2 ml of 2 per cent magnesium sulfate. Add a few drops of chloroform and store in icebox. The stored solution will not deteriorate for several months.

(3) *Ammonium sulfide solution*. Add 2 or 3 drops of the ammonium sulfide solution to a Coplin jar of distilled water.

(4) *Cobalt nitrate solution*. Two per cent aqueous solution.

#### *Procedure*

(1) Suspend organisms in distilled water on microscope slides and allow to dry.

(2) Fix in acetone for 10 minutes.

(3) Rinse thoroughly with distilled water.

(4) Immerse in citrate buffer for 30 minutes.

(5) Rinse thoroughly with distilled water.

(6) Place in the substrate medium overnight.

(7) Rinse thoroughly with distilled water.

(8) Immerse in the cobalt nitrate solution for 5 minutes.

<sup>2</sup> A product of the Eastman Kodak Company (52 per cent alpha- and 48 per cent beta.)

- (9) Rinse thoroughly with distilled water
- (10) Immerse in ammonium sulfide for 1 or 2 minutes
- (11) Rinse thoroughly with distilled water

### *Acid Phosphatase*

#### *Reagents*

- (1) *Citrate buffer* Same as for the alkaline phosphatase
- (2) *Substrate* Combine 6 ml of acetate buffer (30.3 ml of 1 M glacial acetic acid plus 68 ml of 1 M sodium acetate made up to a liter), 5 ml of 0.1 M lead nitrate, 37 ml of distilled water, and 2 ml of 3.2 per cent sodium glycerophosphate<sup>3</sup>. Make fresh each time
- (3) *Ammonium sulfide* Same as for the alkaline phosphatase

#### *Procedure*

- (1) Suspend organisms in distilled water on microscope slides and allow to dry
- (2) Fix in acetone for 10 minutes
- (3) Rinse thoroughly with distilled water
- (4) Immerse in citrate buffer for 30 minutes
- (5) Rinse thoroughly with distilled water
- (6) Place in the substrate medium overnight
- (7) Rinse with distilled water 5 or 6 times at 5-minute intervals
- (8) Immerse in ammonium sulfide for 1 or 2 minutes
- (9) Rinse thoroughly with distilled water

### *Lipase*

#### *Reagents*

- (1) *Citrate buffer* Same as for the alkaline phosphatase
- (2) *Substrate* Stock solution I—Combine 150 ml of 30 per cent glycerol, 50 ml of 10 per cent calcium chloride, 50 ml of barbiturate buffer (29 ml of 0.1 M sodium barbital and 21 ml of 0.1 M hydrochloric acid), and make up to a liter with distilled water

Stock solution II—five per cent aqueous solution of "tween 40" or "60,"<sup>4</sup> or "product 81."<sup>5</sup>

The lipase substrate was prepared for use by adding 2 ml of stock solution II to 50 ml of stock solution I

- (3) *Ammonium sulfide* Same as for the alkaline phosphatase
- (4) *Lead nitrate solution* Two per cent aqueous solution

<sup>3</sup> See footnote 2

<sup>4</sup> Palmitate and stearate, respectively, of a synthetic ester made by the Atlas Powder Company, Wilmington, Delaware

<sup>5</sup> Synthetic stearic acid ester made by the Onyx Oil and Chemical Company, Jersey City, New Jersey

### Procedure

- (1) Suspend organisms in distilled water on microscope slides and allow to dry
- (2) Fix in acetone for 10 minutes
- (3) Rinse thoroughly with distilled water
- (4) Immerse in citrate buffer for 30 minutes
- (5) Rinse thoroughly with distilled water
- (6) Place in the substrate medium overnight
- (7) Rinse thoroughly with distilled water
- (8) Immerse in the lead nitrate solution for 10 minutes
- (9) Rinse thoroughly with distilled water
- (10) Immerse in ammonium sulfide for 1 or 2 minutes
- (11) Rinse thoroughly with distilled water

By means of the King-Armstrong (1934) procedure, quantitative acid and alkaline phosphatase activities were determined on 10 peptone culture suspensions of bacteria for correlation with the phosphatase stains on these organisms

Penicillin was used in a final concentration of 100 units per ml in the substrate solutions employed for the staining reactions. The antibiotic was added at the start of the digestion period. Separate trials showed that the same concentration of penicillin in the culture media stopped the growth of all the organisms tested with the one exception of *Alcaligenes faecalis*. In the quantitative measurements of the phosphatase activity, the effect of penicillin was observed after the culture suspensions were brought to a final concentration of 100 units per ml just before the enzyme measurement was begun.

### RESULTS AND DISCUSSION

The intensity of the phosphatase or lipase stains that were given by the organisms was found to vary with the age of the culture, the older cultures giving weaker stains. Accordingly, 24-hour cultures were employed for all the work included in this report. The effect of magnesium or manganous ions along with alanine in the agar on the intensity of the enzyme staining is illustrated in table 1. It may be seen that certain bacteria that gave negative reactions could be activated to the point of yielding positive stains in some instances.

The organisms used were selected from the museum stock of the Department of Bacteriology, University of Minnesota. It was observed that in many cases various strains of the same organism varied considerably in their enzyme potencies. In some cases one strain of an organism was completely negative in the staining reaction whereas another strain was strongly positive. With the bacteria, both phosphatases did not occur together in the same organism as indicated by the staining. This is not true of certain of the fungi (table 2). It is noteworthy that none of the fungi tested gave a lipase stain, whereas most of the bacteria did.

The lipase stain very rarely revealed definite intracellular morphology. It usually appeared that the enzymatic activity was diffuse, as evidenced by entire areas of golden-brown stain. Very fine strands were thought to be observed on several of the organisms such as *Bacillus indicus* and *Bacillus megatherium*, and a



check on the motility indicated the possibility that the flagella were being stained. Along with the apparent strands noted in these lipase stains another unusual extracellular element was observed. It had the appearance of a spool, and in most cases appeared to be unattached to a cell. The significance of the strands and the spools and of their relationship, if any, to the organisms themselves is not understood.

TABLE 1

*The effect of activators in the medium on the enzyme staining of certain bacteria grown on agar*

ORGANISM	CONTROL AGAR			Mg ALANINE AGAR*			Mn ALANINE AGAR†		
	Alkaline phosphatase	Acid phosphatase	Lipase	Alkaline phosphatase	Acid phosphatase	Lipase	Alkaline phosphatase	Acid phosphatase	Lipase
<i>Klebsiella pneumoniae</i> (strain I)	—	+	—	—	++	—	—	++	+
<i>Aerobacter cloacae</i>	—	—	—	—	—	+	—	—	+
<i>Aerobacter aerogenes</i>	—	+	—	—	+	+	—	+	+
<i>Bacillus mycoides</i>	—	—	+	+	—	+	+	—	+
<i>Bacillus indicus</i>	—	—	+	+	—	+	+	—	+
<i>Alcaligenes faecalis</i>	—	—	—	—	—	—	+	—	+
<i>Klebsiella pneumoniae</i> (strain II)	—	—	—	+	—	±	+	—	±
<i>Bacillus megatherium</i>	—	—	—	—	—	+	—	+	+
<i>Bacillus subtilis</i>	—	—	—	+	—	—	+	—	—

\* Final concentration of magnesium sulfate, 0.1 molar, alanine, 0.017 molar

† Final concentration of manganous sulfate, 0.01 molar, alanine, 0.017 molar

TABLE 2

*Enzyme staining of certain fungi*

ORGANISM	ALKALINE PHOSPHATASE	ACID PHOSPHATASE	LIPASE
<i>Saccharomyces cerevisiae</i>	+	+	—
<i>Saccharomyces fragilis</i>	—	+	—
<i>Candida albicans</i>	+	+	—
<i>Cryptococcus sphaerica</i>	—	+	—
" <i>Torula cremoris</i> "	+	+	—
<i>Cryptococcus</i> species	—	+	—
<i>Geotrichum candidum</i>	—	+	—

Figures 1 to 5 illustrate the appearance of the stained organisms in a few instances. It should be borne in mind that all organisms were treated with citrate buffer of pH 4.7 before the staining reaction was carried out in order to remove extraneous substances that might give a positive test. When the organisms were so treated and then subjected to the staining reagents (but not substrate), no stain was apparent. Thus any stain that these organisms developed later represents enzyme and only enzyme. As another check against false-

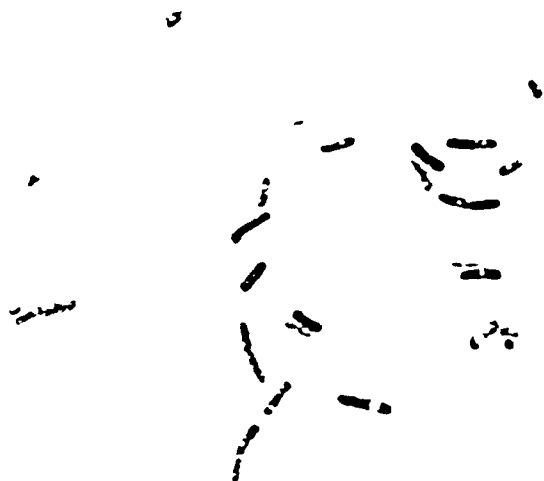
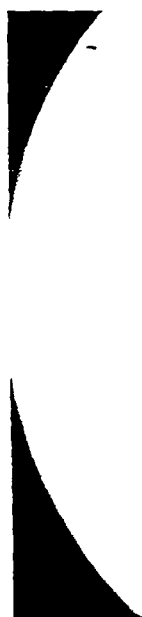


FIG. 1. *BACILLUS MEGATERIUM* STAINED FOR ACID  
PHOSPHATASE.  $\times 1,267$



FIG. 2. *GLOTTRICHUM CANDIDUM* STAINED FOR ACID  
PHOSPHATASE.  $\times 1,267$

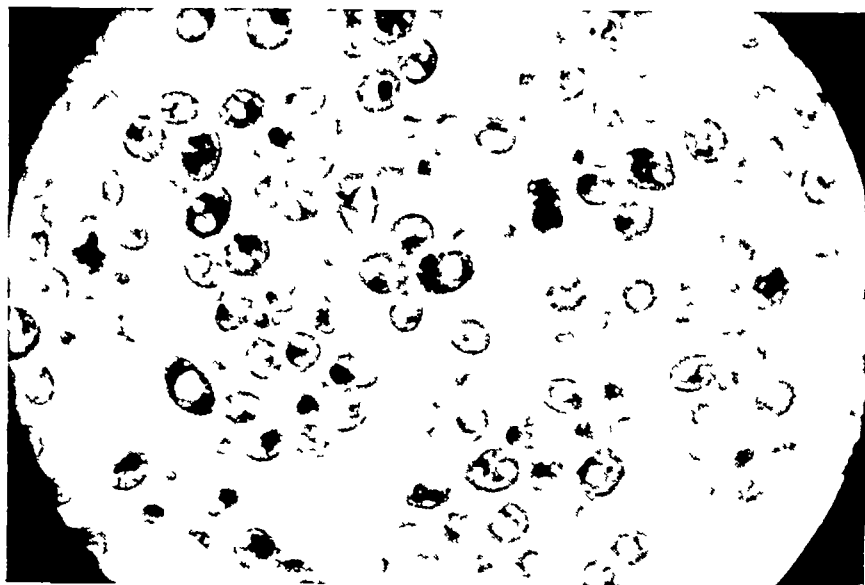


FIG 4 SACCCHAROMYCES CEREVISIAE STAINED FOR ALKALINE PHOSPHATASE  $\times 1,267$

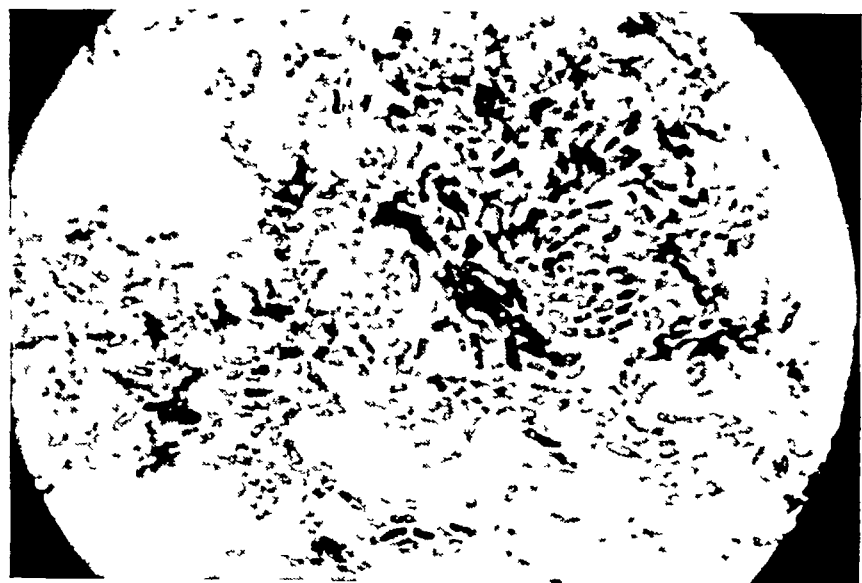


FIG 3 BACILLUS MYCOIDES STAINED FOR ALKALINE PHOSPHATASE  $\times 1,267$

positive reactions, both heat and fluoride were used to inactivate the enzymes. Boiling the fixed preparations for a short time or exposing the slides to a fluoride solution resulted in negative staining in all cases.

From the appearance of electronmicrographs of *Bacillus mycoides*, Knaysi and Baker (1947) reported the presence of nuclei in these organisms. Their photographs showed internal structures very similar to those observed in this investigation under the light microscope after staining for alkaline phosphatase. In

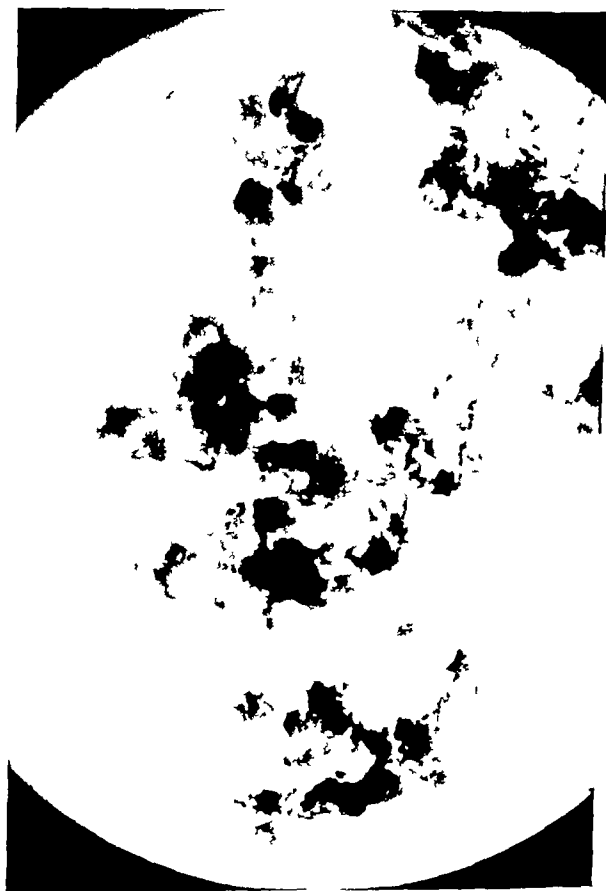


FIG. 5. *BACILLUS SUBTILIS* STAINED FOR LIASE.  $\times 1,267$

both plant and animal tissues it has been shown that phosphatases are present in nuclei. The possibility exists that the stained areas that were observed in the present work may indicate nuclei, but whether or not this is so remains to be proved.

A word of caution should be injected concerning the interpretation of the localized areas of stain. These should not be taken too literally. Ionic and molecular forces are operative that may cause certain deviations in the precipitate pattern and thus lead to a distribution not necessarily identical with the enzyme topography as it existed prior to the staining treatment.

The effect of penicillin on the phosphatase activities of 10 species of bacteria may be seen in table 3. It is obvious that penicillin has little if any effect, even though the concentration used arrested growth in all cases except *Alcaligenes faecalis*. When added to the substrate solutions employed for the enzyme-stain-

TABLE 3

*The effect of penicillin on the phosphatase activities of certain bacterial peptone broth suspensions*

ORGANISM	ALKALINE PHOSPHATASE (UNITS*/100 ML SUSPENSION)			ACID PHOSPHATASE (UNITS†/100 ML SUSPENSION)		
<i>Klebsiella pneumoniae</i> (strain I)						
Control	0	0		7	8	6
Penicillin	0	0		9	2	6
<i>Aerobacter aerogenes</i>						
Control	0	0		9	8	7
Penicillin	0	0		9	0	6
<i>Bacillus mycoides</i>						
Control	18	21	46			
Penicillin	35	20	46			
<i>Bacillus indicus</i>						
Control	11			0	8	
Penicillin	11			0	2	
<i>Alcaligenes faecalis</i>						
Control	0	0	2	0	4	1
Penicillin	17	0	0	8	6	1
<i>Klebsiella pneumoniae</i> (strain II)						
Control	26	13	70	2	0	
Penicillin	28	13	60	2	0	
<i>Bacillus megatherium</i>						
Control	1	0		0	0	2
Penicillin	1	0		2	0	0
<i>Bacillus subtilis</i>						
Control	59	32	37	2	0	
Penicillin	61	29	36	0	0	
<i>Bacillus terminalis</i>						
Control	0	0		1	0	0
Penicillin	0	0		1	2	0

\* Amount of enzyme acting on excess disodium phenyl phosphate at pH 9.0 and 37.5 C that will liberate 1 mg of phenol in 3 hours

† Amount of enzyme acting on excess disodium phenyl phosphate at pH 4.9 and 37.5 C that will liberate 1 mg of phenol in 3 hours

ing reactions, penicillin had no effect on the stains, as would be expected from the quantitative data.

If one compares the data in tables 1 and 3, it is clear that a negative stain does not necessarily indicate the complete absence of enzyme, but merely that the activity is less than some low value. Thus the acid phosphatase stain was negative for *Klebsiella pneumoniae* even though a small degree of activity was present.

## SUMMARY

The Gomori methods for acid and alkaline phosphatases and lipase were employed for the staining of a variety of bacteria and fungi. Localized areas within the organisms gave positive stains in certain instances, suggestive of an internal structure or inhomogeneity.

Manganous and magnesium ions along with alanine in the culture agar were demonstrated to activate the bacterial enzymes and markedly increase their staining intensity.

Penicillin, in concentrations sufficient to arrest completely the growth of certain bacteria, was found to have no significant effect on the phosphatase activities of these bacteria, as demonstrated separately by staining ability and quantitative measurement.

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# THE PRODUCTION OF STREPTOMYCIN BY *STREPTOMYCES BIKINIENSIS*<sup>1</sup> <sup>2</sup>

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Among the various antibiotics produced by actinomycetes, streptomycin occupies a prominent place. So far, the ability to form this substance has been limited to certain strains of organisms belonging to the *Streptomyces griseus* group (Schatz, Bugie, and Waksman, 1944, Waksman, Reilly, and Johnstone, 1946). Most of the other strains of *S. griseus* are unable to produce any antibiotic at all (Waksman, Schatz, and Reynolds, 1946, Carvajal, 1946), or they form other antibiotics, such as grisein (Reynolds, Schatz, and Waksman, 1947). The streptomycin-producing strains of *S. griseus* can easily be differentiated from the nonstreptomycin strains. Two simple procedures can be utilized for this purpose: (1) determination of the sensitivity of the streptomycin strains and the resistance of the nonstreptomycin strains to the action of actinophage (Waksman, Harris, and Reilly, 1948), (2) determination of the resistance of the first and the sensitivity of the second to the action of streptomycin. Sensitivity to streptomycin can be determined by the agar streak method, streaking first a streptomycin-producing strain of *S. griseus* and, after 24 hours' incubation, streaking the unknown strains as test organisms.

The possibility that streptomycin may be produced by organisms other than *S. griseus* has been indicated recently by Trussell, Fulton, and Grant (1947), who isolated a culture of a *Streptomyces* which produced a mixture of antibiotics, one of these appeared to be streptomycin and the other streptothricin. Johnstone and Waksman reported (1947) that a certain organism, tentatively designated as *Streptomyces bikiniensis* and distinct from *S. griseus*, was capable of elaborating an antibiotic that proved to be very similar to streptomycin. Since the chemical identity of this substance with the streptomycin obtained from *S. griseus* was not fully established, the preparation was designated tentatively as streptomycin II. The experimental results dealing with the nature of the organism and with its ability to produce streptomycin form the subject of this paper.

## EXPERIMENTAL PROCEDURES AND RESULTS

*Isolation of organism* In connection with a study of the bacteriological activities in the waters of the Bikini Lagoon and of neighboring areas, carried out during the atomic bomb experiments in July, 1946, several soil samples were taken from the Bikini and Rongelap atolls. The location of these soils, their

<sup>1</sup> Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology

<sup>2</sup> Partly supported by a grant made by the New York Foundation

chemical composition, and their biological properties were reported elsewhere (Johnstone, 1947)

When brought to the laboratory, these soils were plated out, ordinary bacteriological media being used. After certain periods of incubation at 28 C, the plates were examined for the nature and abundance of the various groups of organisms. One of the most striking properties of the microbiological population of those soils was the relatively high percentage of actinomycetes among the colonies developing on the plates. The actinomycetes colonies were picked from the plates and tested for their antagonistic properties by the usual agar cross-streak method. A large number of the cultures, belonging mostly to the genus *Streptomyces*, exerted an inhibiting effect upon the growth of the test bacteria on the agar plate. One of the cultures showed marked inhibition of growth of various gram-positive and gram-negative bacteria. When grown in liquid media, this culture produced an antibiotic that resembled streptomycin in its

TABLE 1

*Inhibition of bacterial growth by actinomycetes as measured by cross streak tests*

STREPTOMYCES SPECIES	TEST BACTERIA			
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Mycobacterium avium</i>	<i>Mycobacterium phlei</i>
	Zone of inhibition in millimeters			
<i>S. bikiniensis</i>	30	21	26	30
<i>S. griseus</i> 4	20	15	13	19
<i>S. griseus</i> 22	20	10	14	19
<i>S. lavendulae</i>	25	20	20	20
<i>Streptomyces</i> G	8	10	14	20
<i>Streptomyces</i> H	20	9	21	22

antibacterial properties or its antibiotic spectrum, in its solubility in water and other solvents, and in various other chemical and biological reactions. Since the culture was, however, distinctly different from *S. griseus*, it was selected for further study. Since it was also different from the other known species of *Streptomyces*, it was decided to designate it as a new species, under the name *Streptomyces bikiniensis*.

The results obtained by the cross-streak agar method (table 1) established the degree of inhibition of various bacteria by *S. bikiniensis*, as compared to similar inhibition of the same bacteria by two strains of streptomycin-producing *S. griseus*, the streptothricin-producing *Streptomyces lavendulae*, and two unknown cultures belonging to the genus *Streptomyces*. *S. bikiniensis* gave on the plate a wider zone of inhibition against the various test bacteria than did the other actinomycetes. When a more complete antibacterial spectrum was made, comparing the relative inhibition of the growth of a number of bacteria, *S. bikiniensis* was found to give a spectrum similar to that of the two strains of *S. griseus*, it was quite distinct, however, from the spectra of the other three actinomycetes.



*Production of streptomycin II* *S. bikiniensis* was grown in the same broth that is commonly used for the production of streptomycin. This broth consists of 3 g meat extract, 5 g peptone, 10 g glucose, and 5 g NaCl per liter of tap water. The cultures were incubated at 28 C, both under static and shaken conditions. Some of the cultures were removed after definite incubation periods, filtered through paper, and the filtrates tested by the agar streak method (Waksman and Reilly, 1945). The results presented in table 2 show that the static cultures were somewhat more satisfactory than were the shaken cultures for the growth of *S. bikiniensis* and for the production of the antibiotic. Although the shaken cultures gave greater activity in a shorter time, this activity never became so high as in the static cultures and soon tended to disappear completely. The

TABLE 2  
*Production of an antibiotic substance by Streptomyces bikiniensis*

INCUBATION	B. SUBTILIS	B. MYCOIDES	E. COLI	S. MARCESCENS
Static cultures				
days				
3	30	10	0	0
6	300	100	10	10
10	300	300	30	30
14	200	200	30	30
Shaken cultures				
1	100	0	0	0
3	100	30	10	10
5	100	100	30	30
7	10	0	0	0

Dilution units per 1 ml of culture

antibacterial spectrum of the culture filtrates appeared in both cases, however, to be very similar to that of streptomycin.

The effect of the nature of the various constituents of the medium and of their concentration upon the production of the antibiotic, *S. bikiniensis* was studied in shaken cultures. The cup method, with a streptomycin standard, was used for testing the activity of the cultures. In the case of glucose, the highest activity was obtained with 0.1 per cent of the sugar, as shown in figure 1. The cultures free from glucose gave as good activity as those containing the higher sugar concentrations. With 2.0 per cent of glucose, the medium remained acid for a long time. This initial acidity was later overcome by the growing culture, the pH rising to its stabilized peak of 8.7. This peak was reached sooner in cultures containing smaller amounts of glucose. The amount of streptomycin produced in 9 days with 2 per cent glucose was only 20 µg per ml, thus showing that glucose has a tendency to delay the formation of streptomycin. Apparently the buffering effect of the glucose, which was found to be necessary in *S. griseus*

cultures, is not essential in cultures of *S bikiniensis*, the highest titers having been obtained in the cultures containing only 0.1 per cent glucose. The pH pattern was the same with the small amount of glucose as with the glucose free culture.

Among the other constituents of the medium, sodium chloride was found to be most effective in 1 per cent concentration. Peptone proved to be a better nitrogen source than tryptone, tryptose, or proteose. Meat extract exerted but

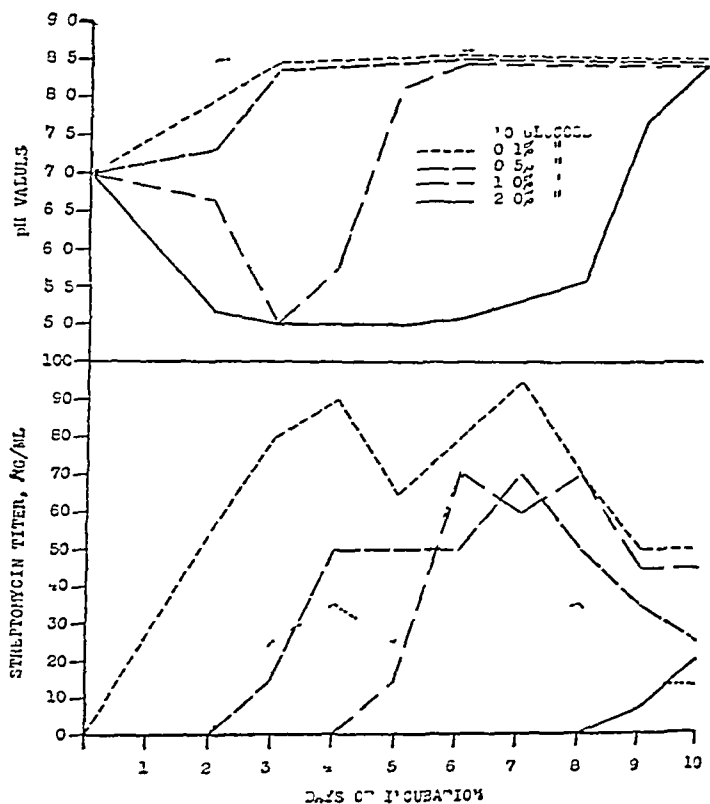


FIG. 1. INFLUENCE OF DIFFERENT CONCENTRATIONS OF GLUCOSE UPON CHANGES IN REACTION AND PRODUCTION OF STREPTOMYCIN BY *S. bikiniensis* IN SUBMERGED CULTURE

little effect upon the production of the antibiotic. This was particularly surprising, since meat extract or its equivalent, such as corn steep or soybean meal, is usually required for the production of streptomycin by *S. griseus*, although synthetic media were recently found to give good yields of this antibiotic. The addition of  $\text{FeSO}_4$  to the medium had no effect upon the production of streptomycin,  $\text{ZnSO}_4$ , however, in concentrations of 50 mg per liter, produced a marked stimulating effect. When both ions were used, the presence of iron tended to neutralize the favorable effect of the zinc.

**Isolation of streptomycin II** The antibiotic produced by *S. bikiniensis* was isolated from the culture medium by the method commonly employed for the

isolation of streptomycin The active material was readily removed from the culture filtrate by adsorption on norit A The adsorbate was washed with neutral alcohol and treated with acidified ethyl alcohol The alcoholic extract was filtered, neutralized, and the alcohol driven off by distillation at low temperatures The residue was dried *in vacuo* The active material could be precipitated directly from the alcoholic solution by the addition of several volumes of acetone It could also be isolated by various procedures commonly used in the isolation of streptomycin, such as the methyl alcohol formic acid method Preparations

TABLE 3  
*Antibiotic spectra of streptomycin and streptomycin II\**

TEST ORGANISM	STREPTOMYCIN	STREPTOMYCIN II
Gram-positive bacteria		
<i>B subtilis</i>	0.3	0.3
<i>B mycoides</i>	1.0	1.0
<i>B megatherium</i>	1.5	1.5
<i>B cuculans</i>	0.3	0.2
<i>B cereus</i>	5.0	5.0
<i>S aureus</i>	5.0	5.0
<i>S lutea</i>	0.1	0.1
<i>M lysodeikticus</i>	1.0	1.0
<i>M avium</i>	0.5	0.5
<i>M phlei</i>	0.05	0.05
<i>M tuberculosis</i> 607	0.3	0.3
Gram-negative bacteria		
<i>E coli</i>	1.0	1.0
<i>S marcescens</i>	3.0	5.0
<i>S alkalescens</i>	9.0	5.0
<i>S paradysenteriae</i>	10.0	10.0
<i>S dysenteriae</i>	5.0	5.0
<i>A aerogenes</i>	3.0	3.0

\* Expressed as micrograms of streptomycin required to inhibit the growth of the organisms in 1 ml of glucose-free nutrient broth

were thus obtained that had an activity of 30 to 50 units per milligram, comparable to the streptomycin yields obtained by similar procedures in the early studies with *S griseus*

The *S bikiniensis* culture was plated out on suitable media, and individual colonies were picked and tested Some of the strains thus obtained were more active in producing the antibiotic than was the original culture By using these isolated strains and improving the method of extraction, preparations were obtained that assayed as high as 158 units per milligram against a streptomycin standard

The streptomycin II preparations were compared to the regular streptomycin for their respective antimicrobial properties The results were reported (table 3) on the basis of micrograms of streptomycin required to inhibit the growth of the

various test organisms. The two antibiotic spectra are identical. In the case of *M. tuberculosis* H37, the amount required for growth inhibition was exactly 21 g per ml for both streptomycin and streptomycin II, as determined by the turbidimetric method (Smith, 1947). These findings were obtained on several repeated tests. The streptomycin-resistant strain of *M. tuberculosis* H37Rv also proved to be resistant to streptomycin II.

In addition to these identical antibacterial spectra, the two preparations showed the following similarities in their action upon other organisms: (1) Both forms of streptomycin were inactive against fungi. (2) Bacteria made resistant to streptomycin were also resistant to streptomycin II. (3) Strep-

TABLE 4  
*Comparative effects of streptomycin and streptomycin II upon Staphylococcus aureus infection in mice*

NO OF MICE	PREPARATION	UNITS PER MOUSE	CULTURE DILUTION	NUMBER OF MICE ALIVE AFTER DAYS				
				1	3	5	7	10
10	Controls	—	10 <sup>-3</sup>	0	—	—	—	—
10	"	—	10 <sup>-4</sup>	2	2	1	1	1
10	"	—	10 <sup>-5</sup>	4	1	—	—	—
10	Streptomycin	5	10 <sup>-3</sup>	1	1	1	1	1
10	"	10	"	3	3	3	3	3
10	"	25	"	9	6	6	6	6
10	"	50	"	10	10	10	10	10
10	"	100	"	10	10	10	10	10
10	Streptomycin II	5	"	1	1	1	1	1
10	"	10	"	3	2	2	2	2
10	"	25	"	9	6	6	6	6
10	"	50	"	10	10	10	10	10
10	"	100	"	10	10	10	10	10

Culture, *S. aureus* SM in 4 per cent mucin, mode of drug administration, subcutaneous, duration of therapy, single dose immediately after infection

tomycin II was inactivated by cysteine, in a manner similar to the inactivation of streptomycin. (4) Both forms of streptomycin gave the same type of reduced activity in the presence of glucose. (5) Both preparations were equally sensitive to increased acidity of the medium.

*Toxicity and in vivo activity of streptomycin II.* Preliminary toxicity tests with streptomycin II, using chick embryos, demonstrated that this preparation when given in large doses—more than 1,200 units per embryo—could be administered with 100 per cent survival.

Samples of the preparation were submitted to the Merck Institute for detailed *in vivo* studies. The results obtained by them emphasized further the remarkable identity in the behavior of streptomycin II with streptomycin.

By courtesy of the Institute, the results of a typical experiment are reported in table 4. Similar effects were obtained when both preparations were used against *Salmonella schottmulleri* in mice.

These results thus prove definitely that *S. bikiniensis*, an organism that belongs to the actinomycetes and that was isolated from a Bikini soil, an organism distinct, both morphologically and culturally, from *S. griseus*, produces an antibiotic that is similar to, if not identical with, streptomycin. Streptomycin II and streptomycin exhibit comparable antibiotic spectra, similarity in physical and chemical properties, and low toxicity to animals. Until the new antibiotic has been crystallized and its clinical activity determined, its absolute identity with streptomycin cannot be established. In view of this, as well as of the minor quantitative differences in the respective antibiotic spectra, and especially of differences in the nature of the organisms producing the two antibiotics, the designation of the newly isolated antibiotic as streptomycin II, namely a streptomycinlike substance, may still be preserved.

#### *Description of Streptomyces bikiniensis*

**Morphology** On glucose asparagine agar, the aerial mycelium arises from the agar surface in the form of single hyphae that subsequently branch heterogeneously. As sporulation develops, these branched mycelia bear straight chains of oval conidia. No tendency to form spirals was noted (figure 2).

**Synthetic agar** (Czapek's). Growth is luxuriant, white, becoming pallid neutral gray (Rdg<sup>3</sup> LIING-f) with white tinge. Aerial mycelium and spores formed abundantly. Superficial droplets, amber-colored. Soluble pigment, light brown.

**Glucose asparagine agar**. Growth luxuriant with good aerial mycelium and spores white, becoming light mouse-gray (Rdg LI 15 ''''Y-O-b). White area in center of slant base remains for about a week, and white fringe remains for a longer period. Superficial droplets, colorless. Soluble pigment, very light amber to no pigment at all.

**Nutrient agar**. Growth luxuriant with moderate aerial mycelium. Color remains white with scanty sporulation. Superficial droplets, none. Soluble pigment, deep brown.

**Nutrient agar plus glucose**. Growth similar to that on nutrient agar, but gray color and spores finally produced. Superficial droplets, amber-colored. Soluble pigment, deep brown, darker than that in nutrient agar.

**Potato plug**. Growth raised and wrinkled, color, pale ochraceous-buff (Rdg VV 15'f), localized and not spreading. Plug, dark brown, local area adjacent to growth, almost black.

**Gelatin**. Liquefaction slight, 1 cm per week at 37 C.

**Nutrient broth**. Growth abundant, complete white surface pellicle formed. Superficial droplets, none. Soluble pigment, deep brown.

<sup>3</sup> Rdg = R. Ridgway, Color standards and color nomenclature. Washington, D. C., 1912.

*Nutrient broth plus glucose* Growth abundant, complete white surface pellicle formed. Aerial mycelium becomes gray in patches. Superficial droplets, amber-colored. Soluble pigment, deep brown.

*Milk* Reaction distinctly alkaline. Coagulation, none. Hydrolysis takes place in 5 days at 28 C. Growth patchy, surface growth of white aerial mycelium becoming gray with sporulation.

*Starch* Growth abundant, white aerial mycelium becoming gray with sporulation. Hydrolysis slight, observable after 3 days at 28 C.

*Habitat* Organisms isolated from a soil obtained from the island of Bikini, Bikini Atoll, in the northern Marshall Islands, during the Bikini bomb experiments in July, 1946. Soil pH 9.0.

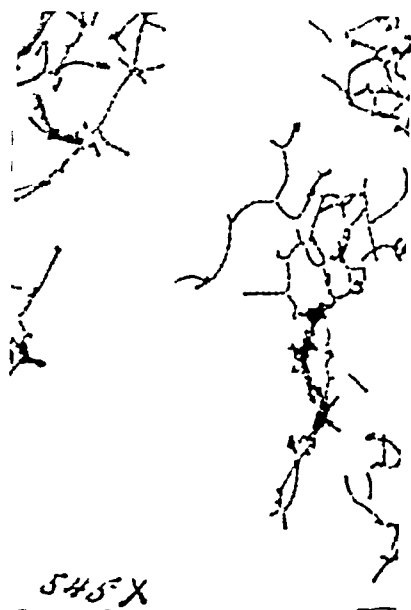


FIG. 2. SPORULATION OF *STREPTOMYCES BIKINIENSIS*.

### *Comparison to Related Species*

The following discussion of related species of the genus *Streptomyces* is based upon a comparison with the earlier work carried out in this laboratory on soil actinomycetes (Waksman, 1919).

*S. bikiniensis* is similar to *Streptomyces aureus* in some of its cultural properties, both produce a gray-colored aerial mycelium on some of the media. On other media, however, even this property is different. *S. aureus* produces a white mycelium on nutrient agar and a cream-colored mycelium on glucose-containing agar, whereas the corresponding pigmentation of the mycelium of *S. bikiniensis* is white and gray. The sporulation of *S. aureus* takes place in the form of spirals in its aerial mycelium, whereas *S. bikiniensis* is completely devoid of spirals in its mycelium on all the media tested, as shown in figure 2.

*Streptomyces olivochromogenus* possesses cultural characteristics that are similar to those of the new culture, with the exception of its growth on potato plug. *S. olivochromogenus* also produces closed spores, whereas *S. bikiniensis* does not.

*S. bikiniensis* resembles *Streptomyces* no. 145 of Waksman (1919) in that it is devoid of spores and produces gray aerial mycelium on glucose-asparagine agar. The latter organism, however, produces gray aerial mycelium and forms no soluble brown pigment on nutrient agar, both of which are contrary to the characteristic properties of *S. bikiniensis*.

*S. bikiniensis* resembles most closely *Streptomyces griseolus* Waksman. Both cultures are similar culturally and morphologically, as shown by a lack of spore formation. The only difference between the two cultures was noted in their growth on potato plug. *S. griseolus* overgrows the plug and produces greenish aerial mycelium, whereas *S. bikiniensis* is limited to localized nonspreading growth on the plug, the color of the growth remaining buff. In addition, the pigmentation of the two cultures on nutrient agar is different; there are also differences in the nature of their growth on glucose asparagine agar, as shown by the following summary.

ORGANISM	GLUCOSE ASPARAGINE AGAR	POTATO PLUG
<i>S. bikiniensis</i>	Surface growth velvety, white border, gray aerial mycelium with colorless droplets of water on surface. Vegetative mycelium white.	Buff to gray colored growth, not spreading. Plug pigmented black.
<i>S. griseolus</i>	Surface growth thin, dry, gray aerial mycelium with no water droplets. Vegetative mycelium dark in color.	Raised growth, buff to gray in color, spreads over whole surface of plug. Plug not pigmented.

Because of these cultural differences, because of the specific nature of the substrate from which the organism was isolated and especially because of the specific physiological properties of the organism producing streptomycin, the authors felt justified in designating this organism as a new species, under the name of *Streptomyces bikiniensis*.

#### SUMMARY

A culture of an actinomycete was isolated from a Bikini soil. This culture grown in an artificial medium, namely, in meat-extract-peptone-glucose broth, produced an antibiotic substance that appeared to be identical with streptomycin. The identity has been established by similar antimicrobial spectra, similar action upon resistant strains of bacteria, similar reactivity with various chemical reagents, and similar effects upon experimental animals. The new streptomycin preparation, however, has not been purified chemically and, as long as its chemical identity with streptomycin has not been established, it has been tentatively designated as streptomycin II, a streptomycinlike antibiotic.

A study of the specific nature of the organism producing streptomycin II

showed it to be markedly different from *Streptomyces griseus* in its morphological and cultural characteristics. It is somewhat similar to *Streptomyces aurcus*, to *Streptomyces olivochromogenus*, and especially to *Streptomyces griseolus*. However, it is not identical with any one of them. Because of this, because of the specific substrate from which it was isolated, and because of characteristic physiological properties that result in the production of streptomycin, it is described as a new species, *Streptomyces bikiniensis*.

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# CHEMICAL ACTIVATION OF ASCOSPORE GERMINATION IN *NEUROSPORA CRASSA*<sup>1</sup>

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In the red bread mold *Neurospora crassa* as in other pyrenomycetes the sexual spores, the ascospores, germinate only after the application of heat (Shear and Dodge, 1927, Goddard, 1935, 1938, 1939) When treated at a temperature of from 50 to 60 C for periods varying from 10 to 60 minutes the spores will germinate up to 100 per cent Germination may occur without this treatment, the percentage depending on the condition of the spores and on the medium used The spontaneous germination rate is usually higher on media fortified with malt and yeast extracts, hay infusion, etc , and may occasionally go as high as for treated spores

Recently, while a synthetic minimal medium was being used in which *d*-xylose was the carbon source, a consistently high percentage of spontaneous germination was observed A number of experiments were then conducted in an attempt to determine the active agent involved From these it appeared that xylose was much more effective after being autoclaved than after being filter sterilized On the assumption that in the mineral solution (Fries no 3, having a pH of 5.5) used in the medium and under the pressure and temperature of autoclaving there might be a slight conversion of the pentose into furfural ( $C_4H_3OCHO$ ), the latter was tried alone and proved very effective

## MATERIALS AND METHODS

The ascospores used in the germination tests were obtained from repeated crosses of wild-type strains E-5256A and E-5297a unless otherwise indicated They were collected in small lots, as they were needed, from the tops of petri dishes in which the crosses had been made, usually from 1 to 4 weeks from the time the perithecia started to shed For germination counts the spores were individually transferred to small blocks of 4 per cent agar in distilled water, flooded with 1.5 per cent sodium hypochlorite solution ("purex"), allowed to stand for a short time until most of the "purex" had drained off, and then transferred to petri dishes containing the medium to be tested The hypochlorite is used to kill any conidia clinging to the ascospores, since conidial germination might obscure that of the ascospores Media used in germination tests consisted of 1.5 per cent agar, 2 per cent sucrose, Fries no 3, and biotin, plus the substance to be tested, which for the sake of uniformity was added after the rest had been autoclaved Experience showed that heat sterilization did not appreciably affect the activity of furfural Controls on the same medium without the added substance were used to measure the germination percentages of each

<sup>1</sup> This represents work supported in part by a grant from the Rockefeller Foundation

lot of spores before and after heat activation. In some cases the chemically treated spores were heat treated, after being counted, to see whether further germination would occur.

TABLE 1

*Effect of furfural concentration on spontaneous germination of ascospores*

FURFURAL CONCENTRATION	SPORES TESTED	GERMINATION WITHOUT HEAT		FURTHER GERMINA- TION ON HEAT TREATMENT	TOTAL GERMINATION		GERMINATED WITHOUT HEAT AS PERCENTAGE OF TOTAL GERMINATED
		no	%		no	%	
0	40	1	2.5	37	38	95.0	2.6
1/1,000	40	38*	95.0				
1/10,000	40	38	95.0				
0	40	0	0	39	39	97.5	0
1/500,000	80	73	91.2				
1/5,000,000	80	12	15.0	66	78	98.7	15.4
1/50,000,000	80	0	0	77	77	96.2	0
1/500,000	100	90	90.0	4	94	94.0	95.7
1/1,000,000	100	85	85.0	13	98	98.0	86.7
1/2,000,000	100	94	94.0	4	98	98.0	95.9
1/4,000,000	100	96	96.0	2	98	98.0	98.0
0	72	0	0	54	54	75.0	0
1/500,000	157	145	92.4	0	145	92.4	100
0	220	2	0.9	182	184	83.6	1.1
1/500,000	139	136	97.8				
1/500,000	100	92	92				
1/1,000,000	100	93	93				
1/2,000,000	100	87	87				
1/4,000,000	100	40	40				
1/8,000,000	100	31	31				
1/16,000,000	100	24	24				
0	320	62	19.4	247	309	96.6	20.1
1/10,000	210	206	98.1				
1/100,000	188	185	97.9				
0	180	1	0.6	173	174	96.7	0.6
1/250,000	204	193	94.6	7	200	98.0	66.5
0	106	11	10.4	82	93	87.7	11.5
1/500,000	100	100	100				100

\* Germination indicated by germinal buds only. At concentrations greater than 1/40,000 furfural is increasingly poisonous to mycelial growth, at 1/1,000 growth is completely inhibited.

## RESULTS

Data from a series of typical experiments are summarized in table 1. A number of trials show that the same degree of activation is brought about by allowing spores to stand from 10 to 15 minutes in a solution of furfural in distilled water, as when a solid medium is used. As can be seen from the table, the germination rate remains consistently high up to dilutions of 1/1,000,000 or more and is in most cases very close to that of the heat-treated spores. With

TABLE 2  
*Effect of degree of maturity of spores upon response to furfural*

CROSS	TYPE AND NUMBER OF SPORES	MEDIUM	GERMINATION WITHOUT HEAT		FURTHER GERMINATION ON HEAT TREATMENT	TOTAL AS PER CENT
			no	%		
Abb-4A	135 shed spores	1/100,000 furfural	122	90.4		
and E-5297a	8 asci (59 spores)	1/100,000 furfural	6	10.2		
Abb-4A	145 shed spores	1/100,000 furfural	115	79.3		
and 25a	6 asci (42 spores)	1/100,000 furfural	5	11.9		
Abb-4A	50 shed spores	1/100,000 furfural	44	88.0	3	94.0
and E 5297a	9 asci (65 spores)	1/100,000 furfural	16	24.6	39	84.6
E 5256A	96 shed spores	Minimal	9	9.4	71	83.3
	106 shed spores	1/500,000 furfural	92	86.9	7	93.2
and E 5297a	12 asci (88 spores)	1/500,000 furfural	25	28.4	48	83.0

greater dilutions the rate drops fairly fast, and there is greater variation between spore lots. The latter may be due partially to age differences, since the age or ripeness of spores is of more critical importance in chemical than in heat activation. This is most noticeable in the case of spores dissected from asci. Table 2 gives a comparison in several crosses of the difference in germination rate on furfural between spores already shed and those from ripe asci (as judged by the color of the spores and the fragility of the ascus sac). There would be very little or no difference if the two sets were activated by heat.

Goddard has shown that in *Neurospora tetrasperma* heat activation of asco-

spores is reversible. This does not seem to be true of chemical activation by furfural. Neither furfural activation nor heat activation is sensitive to cyanide ( $M/1,000$  or  $M/500$ ), but the respiration of the activated spore is, and prolonged immersion in cyanide not only inhibits nearly all growth but reverses the activation process. The originally heat-activated spores can be again activated by further heating, and this cycle can be repeated as desired. On the other hand, spores that have once been activated by furfural and then induced to return to dormancy by the cyanide treatment become refractory to further furfural treatment but remain sensitive to heat activation. In one experiment spores that had been returned to dormancy after heat treatment were reactivated by furfural, but this could not be repeated in two other trials.

Other furan derivatives and other aldehydes are being tested for activity. Of the substances in the first group so far tested (furan, furfuryl alcohol, furoic acid, furoamide, furoin,  $\beta$ -furfural dioxime) only furfuryl alcohol was active. When used immediately after purification, it seems to be about 75 per cent as effective as furfural. On standing, a considerable portion is converted to the aldehyde and the test loses meaning.

No definite report can yet be made on other aldehydes except to say that the one that might most easily be expected to be the naturally occurring agent, acetaldehyde, has been entirely negative in every test conducted so far. Nor has it been possible to demonstrate the presence of furfural during heat activation, though chemical tests are sensitive for concentrations as low as 1 part in 5 to 10 million.

It must remain an open question whether furfural is a natural agent in inducing ascospore germination. The environments in which *Neurospora* is usually found might easily have traces of furfural, and possible precursors of furfural are doubtless present within the ascospores, but there is no direct evidence that either plays a role in ascospore germination in nature.

#### SUMMARY

Furfural is shown to be an effective chemical agent for activating the dormant ascospores of *Neurospora crassa*. The germination rate of chemically activated spores approximates that brought about by heat treatment up to dilutions of  $1/1,000,000$ , with greater dilutions the rate drops sharply, but variably. The high germination rates apply only to spores already shed, those dissected from asci are recalcitrant to chemical activation.

# PHYSIOLOGICAL STUDIES ON SPORE GERMINATION, WITH SPECIAL REFERENCE TO CLOSTRIDIUM BOTULINUM

## III CARBON DIOXIDE AND GERMINATION, WITH A NOTE ON CARBON DIOXIDE AND AEROBIC SPORES<sup>1</sup>

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The requirement of CO<sub>2</sub> for vegetative cell development of bacteria is common knowledge and needs no review here, but scarcely anything is known of the relation of CO<sub>2</sub> to the process of bacterial spore germination as distinct from subsequent vegetative development. One might consider the latter in the sense of transition from heat-stable to heat-labile form (Wynne and Foster, 1948a). Relevant is the incidental observation that spores of one out of three strains of *Clostridium botulinum* failed to produce colonies in 72 hours when incubated in a vacuum (Morrison and Rettger, 1930).

It is rather common to discover that special efforts to eliminate CO<sub>2</sub> from the culture system, and to minimize the formation of CO<sub>2</sub> by the cells in the inoculum by supplying a low nutrition level medium, result in a retardation of growth that may extend indefinitely.

Our study of factors determinant in the germination process itself (as distinct from subsequent vegetative development) of *Clostridium botulinum*, begun in two previous papers (Wynne and Foster, 1948a, b), has included examination of the CO<sub>2</sub> effect. This stems from the finding that anaerobiosis secured by alkaline pyrogallol seems to delay germination of botulinum spores. Background information and general methodology are covered in the first of these papers and need not be reiterated here. To secure anaerobic conditions free of CO<sub>2</sub>, vacuum desiccators containing the culture tubes were evacuated with a Cenco Hyvac pump for 30 to 60 minutes and refilled with natural (illuminating) gas (CH<sub>4</sub>) cleansed of CO<sub>2</sub> by slow passage through a gas-washing train consisting of three bottles of NaOH and one of N/10 Ba(OH)<sub>2</sub>. The latter was second last in the chain, functioning as a CO<sub>2</sub> indicator. As an added precaution normal NaOH was always placed in the bottom of the desiccator. Where a CO<sub>2</sub> atmosphere was required, it was added from a cylinder or generated in the desiccator by mixing excess acid with the calculated amount of solid NaHCO<sub>3</sub>. Unless otherwise specified germination always took place in Difco brain-heart infusion broth with BBL thioglycolate supplement, and always the inoculum was about 500 spores per ml of medium. Table 1 compares the spore germination in atmospheres con-

<sup>1</sup> This project has been undertaken in co operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or having the endorsement of the War Department.

taining 0, 1, and 5 per cent CO<sub>2</sub>. The CO<sub>2</sub> effect is striking. Germination is negligible in the absence of CO<sub>2</sub>, whereas almost all the spores germinated in the presence of CO<sub>2</sub>, the higher CO<sub>2</sub> tension being somewhat better. The difference between the two CO<sub>2</sub> treatments actually was greater than it appears, turbidity developed in 15 hours in the 5 per cent CO<sub>2</sub> desiccator and in 19 hours in 1 per cent CO<sub>2</sub>. No turbidity appeared in the zero CO<sub>2</sub> control at 22 hours, the termination of the experiment.

However, a CO<sub>2</sub> effect could not be obtained for four other species of anaerobic sporeformers tested similarly *Clostridium chauvei*, *Clostridium histolyticum*, *Clostridium perfringens*, and the well-known food spoilage organism designated as putrefactive no. 3679. This was true even at pH 6.0, which was chosen to reduce the solubility of CO<sub>2</sub> in the medium and which was the lowest pH supporting germination of these anaerobes. Thus under identical conditions germination of *C. botulinum* spores is inhibited by lack of CO<sub>2</sub>, and germination of

TABLE 1  
*Effect of CO<sub>2</sub> concentration on germination*

INCUBATION	CO <sub>2</sub> TENSION	RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
hr	%			%
0	—	560	—	—
22	0	520	40	7
22	1	90	470	84
22	5	18	540	97

the other four anaerobes is not. A possible interpretation of this is given in the discussion below.

The *C. botulinum* experiment described above (see also table 1) demonstrates only a rate effect under the conditions used, for whereas only 7 per cent germination occurred in the CO<sub>2</sub>-free control at the 22-hour period, prolongation of incubation always resulted in high germination and pronounced turbidity. Failure to demonstrate an absolute CO<sub>2</sub> effect in brain-heart medium, even with several painstaking experiments involving modification of pH, exhaustive pumping, omission of the colloidal agar of the anaerobic supplement, continuous gassing with N<sub>2</sub>, etc., was considered on two counts to be probably tied up with the complex nature of the brain-heart medium. (1) The CO<sub>2</sub> effect can be accentuated by eliminating complex media in favor of synthetic (Gladstone, Fildes, and Richardson, 1935) or by employing complex media at a minimal nutritional concentration, i.e., with respect to carbohydrate and protein content (Rockwell and Highberger, 1926, 1927). The first count may be considered to anticipate the issue of the second. (2) CO<sub>2</sub> should be dispensable so long as certain organic substances are present in whose synthesis CO<sub>2</sub> participates. The presence of such substances is likely in complex media of biological origin, and in such media, therefore, the need for CO<sub>2</sub> should be obviated. While our attack on these lines

was under way other reports appeared that confirm the logic of this approach ( $C_4$  dicarboxylic acids, Ajl, White, and Werkman, 1947, aspartic acid, Lardy *et al.*, 1947, Lardy, 1947)

A synthetic medium similar to that devised by Roessler<sup>2</sup> (1946) for the growth of *C. botulinum* was used as a starting point, but with only one-tenth the regular concentration of amino acids (Results were similar, however, with the full medium, which contained 1 per cent amino acids) This medium supported abundant vegetative development of our strain of *C. botulinum*, and spore germination was much slower than in complex media, seemingly opening an approach to factors essential for germination, including those involving  $CO_2$  and those not For good anaerobic growth it was expeditious to add 0.2 per cent glucose to the synthetic media, as germination is negligible in its absence

With an inoculum of around 500 spores per ml, clear-cut turbidity developed in the presence of  $CO_2$  at about 72 hours, but counts at 87 hours showed that

TABLE 2

*Quantitative indefinite inhibition of germination of C. botulinum spores due to the absence of  $CO_2$*

$CO_2$ IN ATMOSPHERE	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
%	days			%
—	1	560	—	—
0	17	500	60	11
1	5	220	340	61

\* Counts corrected for volume loss of 9 per cent during prolonged incubation over NaOH. No turbidity developed in any of the tubes in this series

only about 15 per cent of the spores had germinated In subsequent work a 5-day incubation period was employed for positive  $CO_2$  controls, for in this time germination counts were well over 50 per cent In such a medium it is possible to approach an absolute  $CO_2$  requirement for germination Thus in an experiment in which the positive (1 per cent)  $CO_2$  control showed 61 per cent germination in 5 days, the  $CO_2$ -free treatment showed only 11 per cent germination and no turbidity even after 17 days (table 2) Indeed, the figure of 11 per cent may not be significant at all owing to the fact that the spore-counting method has an over-all accuracy of  $\pm 9$  per cent and occasionally has spread wider than this (Wynne and Foster, 1948a) Under these conditions clear-cut turbidity always

<sup>2</sup> This medium had the following composition: *dl*-leucine, 0.0083 M, *dl*-phenylalanine, 0.0132 M, *l*-arginine, 0.0065 M, *dl*-valine, 0.0083 M, *dl* isoleucine, 0.004 M, *l* tryptophane, 0.11 M, *l* tyrosine, 0.0003 M, *dl* methionine, 0.002 M, *dl*-threonine, 0.0067 M, *dl* serine, 0.01 M, *l*-histidine, 0.0013 M, biotin, 5  $\mu$ g per ml, PABA, 0.02  $\mu$ g per ml, nicotinamide, 1  $\mu$ g per ml, thiamine, 0.2  $\mu$ g per ml, yeast nucleic acid, 0.01 per cent, Na thioglycolate, 0.05 per cent,  $MgSO_4$ , 0.0002 M,  $MnSO_4$ , 0.0001 M,  $CaCl_2$ , 0.0001 M,  $FeSO_4$ , 0.00005 M, K  $HPO_4$ , 0.015 M,  $KH_2PO_4$ , 0.015 M

follows germination within a few hours. Thus probably no germination at all occurred in the CO<sub>2</sub>-free tube, the 11 per cent value doubtless being an experimental counting error.

It seems safe, therefore, to conclude that CO<sub>2</sub> is absolutely essential for spore germination of *C. botulinum* in a medium otherwise adequate for that process. This apparently is the first demonstration of CO<sub>2</sub> requirement specifically for the germination process, and apart from subsequent vegetative development.

TABLE 3  
Effect of oxalacetate on germination in CO<sub>2</sub>-free gas phase  
Experiment A

INCUBATION	CO <sub>2</sub> IN ATMOSPHERE	OAA 10 <sup>-3</sup> M	EXHAUSTION PERIOD	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
hr	%		minutes			%
0	—	—		535	—	—
20	0	—		460	75	14
20	1	—		340	195	36
20	0	+		74	460	86

Experiment B

0	—	—	—	520	—	—
23	0	0	30	470	50	10
23	0	+	30	35	485	93
23	0	+	270	65	455	88
23	1	0	30	21	500	96

#### BY-PASSING CARBON DIOXIDE

*Oxalacetic acid* Along the lines discussed under count (2) above, the C<sub>4</sub> dicarboxylic acids were tested for their ability to permit germination in the absence of CO<sub>2</sub>, as the universality of the Wood-Werkman reaction via pyruvate fixation of CO<sub>2</sub> indicates the likelihood of their being involved here. The primary fixation product, oxalacetic acid (OAA), is generally in biological equilibrium with malic, fumaric, and succinic acids, all vital catalysts or intermediates in cells. OAA in brain-heart media definitely promotes the germination rate of spores in the absence of gaseous CO<sub>2</sub> (table 3) and apparently by-passes CO<sub>2</sub>. Experiment A in table 3 shows that the OAA induced spore germination at a rate appreciably faster than a 1 per cent CO<sub>2</sub> gas tension, and in experiment B it was equal to the CO<sub>2</sub> in promoting germination. The chances are that OAA would have induced faster germination in experiment B also had the counts been made at a shorter incubation period.

The OAA effect might, to a certain extent, be ascribed to CO<sub>2</sub> resulting from the spontaneous decomposition of OAA to CO<sub>2</sub> and pyruvic acid (Krampitz and Werkman, 1941, Krebs, 1942). OAA in solution at 37 C has a very short half-life and its decomposition is catalyzed by amino groups and by traces of cationic



metals. However, since OAA gives a germination rate exceeding that of  $\text{CO}_2$ , the effect seemingly is due to the OAA per se, though  $\text{CO}_2$  may contribute to the rate partially. Germination by OAA was not retarded when the medium was continuously exhausted with a Hyvac pump for 4.5 hours after OAA addition, the idea being to remove quickly any  $\text{CO}_2$  generated from OAA (exp. B, table 3). As no lessening of the OAA effect by this continuous  $\text{CO}_2$  removal was observed, the probability of a direct OAA participation seems good.

Maybe a brief contact with  $\text{CO}_2$ , such as would occur in the pumping experiment mentioned above, would suffice for germination, but other experiments showed that contact with a 1 per cent  $\text{CO}_2$  atmosphere for the initial 4 hours, followed by removal ("hyvac") and replacement with  $\text{CO}_2$ -free gas had an insignificant effect on germination.

*Stable C<sub>4</sub> dicarboxylic acids* A mixture of *l*-malic, fumaric, and succinic acids (Na salts), each at a concentration of  $3.3 \times 10^{-4} M$ , was shown repeatedly to have a definite acceleration on germination rate in the absence of  $\text{CO}_2$ . These acids were not as effective as  $\text{CO}_2$  (or OAA) in promoting germination. The efficacy of these acids in promoting spore germination was roughly about one-third that of a 1 per cent  $\text{CO}_2$  gas phase. The inability of the acids to substitute fully for OAA has been encountered previously (Shive and Rogers, 1947, and others) and probably relates to membrane penetration at pH values in physiological range, in which these acids are almost 100 per cent dissociated. It will be recalled that OAA itself does not penetrate unaltered cells of *Micrococcus lysodeikticus* (Krampitz and Werkman, 1941), and several other examples could be given. If these acids diffuse in the molecular (undissociated) form as do the free acids, it would be expected that diffusion would be greatest at pH 3 to 4, as the acids are almost entirely in molecular form in this range as contrasted to a negligible percentage at pH 6 or above. It was not possible to test this with *C. botulinum*, as germination is inhibited at pH values below 6. It will be recalled that Ajl, White, and Werkman (1947) found that the C<sub>4</sub> dicarboxylic acids or their respiratory precursors by-passed the  $\text{CO}_2$  requirements for coliform bacteria.

The specificity of the effect for the C<sub>4</sub> dicarboxylic acids on *botulinum* germination is exemplified by the fact that no demonstrable action was given by  $\alpha$ -ketoglutarate, glutarate, valerate, butyrate, propionate, lactate, or pyruvate. On the other hand, a striking stimulation in vegetative development was induced by all these acids (except pyruvic) at  $10^{-3} M$ . So marked was this that cultures with well-advanced turbidities showed surprisingly small germination percentages. This is a fine example of the fallacy of judging germination rates by the intensity of vegetative turbidity.

*Aspartic acid* As OAA is converted to aspartic acid by transamination, one might expect that this amino acid also would by-pass the  $\text{CO}_2$  requirement, the latter participating in the synthesis of aspartate. This has indeed been demonstrated for *Lactobacillus arabinosus* (Lardy *et al.*, 1947, Lardy, 1947), in which case aspartate is apparently the only constituent of cell material in the synthesis of which  $\text{CO}_2$  participates, excepting perhaps for relatively insignificant amounts

of other components This was proved by isotopic  $\text{CO}_2$ , substantially the entire content of the labeled C in the cells being in the carboxyl groups of the cellular aspartate It is likely that the other  $\text{C}_4$  dicarboxylic acids are converted to aspartate via OAA

The germination tests were conducted in Roessler's synthetic medium (1/10 strength amino acids) which, as a basal medium, lacked  $\text{NaHCO}_3$ , biotin, and aspartic acid Preliminary experiments indicated that neither biotin + aspartate nor sodium oleate + aspartate could by-pass  $\text{CO}_2$  (Oleate was tested because of its known biotin-sparing action) Mixtures of biotin (5  $\mu\text{g}$  per ml), aspartate ( $10^{-3}$  M or  $10^{-4}$  M), and oleate (1, 10, or 100  $\mu\text{g}$  per ml) were also tested, but germination was insignificant in the absence of  $\text{CO}_2$  after 14 days' incubation

TABLE 4  
*Effect of complex supplements on germination*

INCUBATION	1% $\text{CO}_2$	SUPPLEMENT ADDED	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
days					%
0			450		
1	+	1% yeast extract	15	435	97
1	+	1% liver extract	230	220	49
1	+	1% brain heart	35	415	92
2	—	1% yeast extract	45	405	90
2	—	0 1% yeast extract	120	330	73
2	—	1% liver extract*	385	65	14
2	—	1% brain heart*	50	400	89
5	+	None	160	290	64
15	—	None	425	25	5
15	—	1% liver extract†	475	0	0
15	—	0 1% liver extract	475	0	0
15	—	1% brain heart†	430	20	4
15	—	0 1% brain heart	410	40	9

\* One out of three tubes, of which † represents remaining two

tion The control medium in the presence of  $\text{CO}_2$  showed 50 per cent germination at 7 days

Finally, the following known or suspected by-passing substances and available participants in the tricarboxylic acid respiratory system were tested in combination, all at  $10^{-3}$  M in basal synthetic medium, in the presence and in the absence of  $\text{CO}_2$  aspartate, malate, fumarate, succinate,  $\alpha$ -ketoglutarate, glutamic acid, glutarate, and *cis*-aconitate These were entirely unsuccessful in by-passing  $\text{CO}_2$  When  $\text{CO}_2$  was present, the germination rate was unaffected in this medium, a fact indicating no toxicity caused by the supplements

*Complex supplements* Also, the following complex organic supplements were tested in triplicate tubes at 0.1 and 1.0 per cent levels in the basal synthetic medium, again in the absence and in the presence of  $\text{CO}_2$  brain-heart infusion, liver extract,<sup>3</sup> and yeast extract, all Difco The  $\text{CO}_2$ -free yeast and liver treat-

<sup>3</sup> Extract of 0.1 and 1.0 per cent dried liver

ments were incubated in one desiccator, the CO<sub>2</sub>-free brain-heart in another, and the CO<sub>2</sub>-free synthetic medium in another. Within 40 hours in the absence of CO<sub>2</sub> all the yeast tubes, a single 1 per cent liver tube, and a single 1 per cent brain-heart tube developed marked turbidity. To avoid contaminating the other tubes with fermentation CO<sub>2</sub>, these turbid tubes were removed, pasteurized, and held for spore counts. All the tubes of synthetic medium in CO<sub>2</sub> showed turbidity at 3 to 4 days and were removed for counting on the fifth day. The CO<sub>2</sub>-free synthetic medium showed no turbidity even after 15 days, the termination of the experiment, and the remaining liver and brain-heart tubes in CO<sub>2</sub>-free atmosphere behaved similarly. Residual spore counts for this experiment are in table 4.

It is clear that yeast contains CO<sub>2</sub> by-passing factor (s) that are not identical with the supplements added to the basal medium, because CO<sub>2</sub> was necessary for germination in the latter treatment but not in the yeast. Yeast apparently is richest in the unknown by-passing factor(s), as the liver and the brain-heart were greatly inferior in this respect. The rapid growth in the yeast tubes in the same desiccator as the negative liver tubes shows that the effect resides specifically in their contents of CO<sub>2</sub> by-passing substances and not in a CO<sub>2</sub> leak or other artifact leading to the unintentional presence of CO<sub>2</sub>, for the smallest amounts of CO<sub>2</sub> induce rapid germination in the liver (and brain-heart medium). One will note that even the amounts of CO<sub>2</sub> generated by the turbid yeast tubes were insufficient to induce significant germination in brain-heart medium.

#### AEROBIC SPOREFORMERS

Some testing of a survey nature was done with four species of aerobic spore-formers: *Bacillus brevis*, *Bacillus megatherium*, *Bacillus mesentericus*, and *Bacillus subtilis*. Germination occurred in Difco nutrient broth in shallow layers in 50-ml Erlenmeyer flasks at room temperature. CO<sub>2</sub>-free treatments were conducted in desiccators with air as the gas phase. In no case was it possible to retard germination of these organisms in a CO<sub>2</sub>-free atmosphere. Evidently this is due to the presence in the nutrient broth of organic substances by-passing the CO<sub>2</sub>, although no attempt was made to confirm this with synthetic media. Interestingly enough, though CO<sub>2</sub> did not enhance the germination of any of these four aerobes, in one, *B. mesentericus*, the stable C<sub>4</sub> dicarboxylic acids mixture ( $3.3 \times 10^{-4}$  M each) distinctly accelerated the germination. Thus in a typical experiment with an inoculum of 3,040 spores per ml, 29 per cent germination was obtained in the CO<sub>2</sub>-free treatment after 25 hours and 74 per cent in the C<sub>4</sub> treatment. This organism presumably was inefficient in the conversion of CO<sub>2</sub> to C<sub>4</sub> dicarboxylic acids.

The behavior of each of these aerobes in respect to vegetative development in relation to CO<sub>2</sub> is in decided contrast to that of the spores, for with each aerobe CO<sub>2</sub> induced a marked acceleration. This again emphasizes the distinction between the germination process and the subsequent vegetative activity of sporeforming bacteria.

## DISCUSSION AND SUMMARY

The germination process and vegetative cells are not affected alike by CO<sub>2</sub> and the C<sub>4</sub> dicarboxylic acids. The fact that germination in four out of five anaerobes tested failed to respond to CO<sub>2</sub>, whereas in *Clostridium botulinum* it did, indicates species or strain differences. This applied also to the differences described for the four aerobic sporeformers.

A clue to the nature of these effects comes from the fact that in complex media (i.e., brain-heart infusion) CO<sub>2</sub> deprivation only slowed down the rate of germination but did not stop it, whereas in a synthetic medium germination could be entirely suppressed indefinitely without CO<sub>2</sub>. Judging from the evidence available, this could mean that present in complex media are substances as yet unknown that can by-pass CO<sub>2</sub>. Some such substances are known (see above, C<sub>4</sub> dicarboxylic acids and aspartic acid), but these could not substitute for CO<sub>2</sub> in the germination of *C. botulinum* spores in a synthetic medium that is otherwise adequate for germination and growth. Does this mean, then, that present in complex natural materials are additional new substances capable of by-passing the CO<sub>2</sub> requirement, in whose synthesis CO<sub>2</sub> participates when they are not supplied artificially to the medium? Seemingly the positive germination results obtained in a CO<sub>2</sub>-free system upon addition of small amounts of yeast extract to the basal synthetic medium speak in this behalf. After this work was completed, the recent report of Lwoff and Monod (1947) was received. These authors, working with *Escherichia coli*, found C<sub>4</sub> and C<sub>5</sub> dicarboxylic acids and their amino derivatives to be effective CO<sub>2</sub> by-passing agents, but that they alone did not suffice, and they came to exactly the same conclusions as those given above namely, other essential CO<sub>2</sub> by-passing agents are present in complex natural materials.

One may suspect that CO<sub>2</sub> is involved in the synthesis of biological substances other than C<sub>4</sub> and C<sub>5</sub> acids and the derived aspartic and glutamic acids, and, indeed, at least one other system is already known, viz., carboxylation of  $\alpha$ -ketoglutaric acid to oxalosuccinic acid (Ochoa, 1945). Others are under suspicion, and new ones not only are a distinct possibility but on the basis of the foregoing evidence must exist.

Variations in response to C<sub>4</sub> dicarboxylic acids mean that these are required in different degrees by different organisms. Thus, the response by *C. botulinum*, in the complex medium, to added C<sub>4</sub> acids indicates that these were limiting or near limiting in germination. Complete lack of response to CO<sub>2</sub> by the other clostridia indicates that whatever by-passing agents (presumably including the C<sub>4</sub> acids) were present they were sufficient to by-pass CO<sub>2</sub> entirely. Similar differences showed up in the aerobes though removal of CO<sub>2</sub> did not retard germination in any of the four species, C<sub>4</sub> acids significantly stimulated germination in *Bacillus mesentericus* and were therefore limiting even in the presence of CO<sub>2</sub>. It is possible that the C<sub>4</sub> acids may fully by-pass CO<sub>2</sub> in this organism. If C<sub>4</sub> acids play a role in the by-passing of CO<sub>2</sub> in the other three aerobes, the concentration present in nutrient broth must be adequate, though other substances may be involved.

The main conclusion obtainable from all these observations is that organisms differ widely in the extent to which medium components enable them to by-pass their CO<sub>2</sub> requirements and that some hitherto-unrecognized CO<sub>2</sub> by-passing substances exist. A corollary is that a complete diet of organic compounds renders CO<sub>2</sub> dispensable for germination and initiation of growth.

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# THE RELATIVE ANTISYPHILITIC ACTIVITY OF PENICILLINS F, G, K, AND X AND OF BACITRACIN, BASED ON THE AMOUNTS REQUIRED TO ABORT EARLY SYPHILITIC INFECTIONS IN RABBITS

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The assay of antisiphilic agents by the determination of their curative dose in infected rabbits is a laborious and time-consuming procedure. The disappearance of organisms from the primary lesion is an unreliable criterion of cure, since the treponemata may reappear days, weeks, or even months after treatment, and since a large proportion of the rabbits in which the primary lesion is permanently healed are nevertheless found to harbor spirochetes in the lymph nodes at some later date. The injection of a lymph node emulsion into a normal animal may itself be an unreliable index of cure if it is carried out too soon after treatment, since the nodes may be only temporarily rid of treponemata. Thus, a popliteal lymph node may be noninfectious 6 weeks after treatment, but the second node may be infectious if tested 4-6 months later (Eagle, Hogan, and Kemp, 1942), nor indeed is there absolute assurance that even after 6 months residual organisms have always remultiplied sufficiently to make the lymph nodes regularly infectious.

Rake, Dunham, and Donovan (1947) have recently suggested a method of evaluating antisiphilic agents that promises to reduce materially the time necessary for their evaluation. In brief, the method is based on the fact (Magnuson and Eagle, 1945, Eagle, Magnuson, and Fleischman, 1947) that a relatively minute amount of treatment suffices to terminate syphilitic infection in rabbits if it is administered soon after inoculation, and before the appearance of the primary lesion. At least with mapharsen and penicillin, asymptomatic infection under these conditions is so uncommon that it may safely be ignored as a complicating factor, and doses that prevent the appearance of the primary lesion usually have actually aborted the infection (Magnuson and Eagle, 1945, Eagle, Magnuson, and Fleischman, 1947, and footnote to table 2). The relative antisiphilic activity of a series of compounds can therefore be determined in a matter of 2 to 3 months instead of 9 to 12 months. A qualification is, however, necessitated by the fact that, since the animals had been treated before the development of lesions, the relative abortive activity of a series of compounds

<sup>1</sup> One unit of bacitracin, as defined by Johnson, Anker, and Meleney, is that "amount which when diluted 1:1024 in a series of two-fold dilutions in 2 cc. of beef infusion broth, completely inhibits the growth of a stock strain of Group A hemolytic streptococcus when the inoculum used to seed the tubes is 0.1 cc. of a 10<sup>-2</sup> dilution of an overnight culture in blood broth."

TABLE 1

Available information with respect to the relative antisyphilitic activity of penicillins F, G, K, and X and of bacitracin

	EFFECT ON REITER STRAIN OF T PALLIDUM IN VITRO	EFFECT ON PATHOGENIC T PALLIDUM IN VIVO								
		Conc necessary for complete inhibition of growth (Eagle 1946 1948)	Dosages necessary to cause disappearance* of organisms (Turner Cumberland, and Li)		Dosages that cure established infection†		Dosages that abort infection if given during incubation period			
							Rake, Dunham, and Donovan‡		Present experiments§ (see table 3)	
		mg/kg	units/kg	mg/kg	units/kg	mg/kg	u/kg	mg/kg	u/kg	
Penicillin F G K X	0.025-0.1 micrograms/ml in individual experiments	0.63	1,000	9.4, 14	8,500, 12,700	0.20	310	3.5	53	
		0.11	185	0.87, 0.96	1,450, 1,600	0.065	109	0.3	5	
		>2	>4,400	7, >7	16,000, >16,000	0.28	645	2.6	60	
		1.2	1,050	?	?	0.19	173	2.2	20	
Bacitracin	0.004 units/ml		72¶	?	?	—	—		90	

Relative activity per mg, referred to that of penicillin G as 100

Penicillin					
F	53	18	9.2, 6.2	31	8
G	100	100	100	100	100
K	76	<5.5	8, <8	23	11.5
X	51	9	—	34	14
Bacitracin	33	5¶	—	—	10

\* Actually, the dosage necessary to reduce the number of organisms in the chancre to 10 per 200 microscopic fields, when administered intramuscularly 3 times at 2 hour intervals

† Twenty-four intramuscular injections at 4-hour intervals administered 6 weeks after intrastriatal inoculation with several million organisms (Arnold, Boak, Carpenter, Chesney, Fleming, Gueft, Mahoney, and Rosahn, 1947). The 2 values in the table represent results obtained in different laboratories

‡ Twenty-four intramuscular injections at 4 hour intervals, beginning 6 hours after intradermal inoculation with 60,000 organisms

§ Single intramuscular injection daily for 4 days, beginning 4 days after intradermal inoculation with 2,000 organisms

|| Activity relative to penicillin G of a crude preparation assaying at 30 units per kg. A more highly refined product would be correspondingly more active

¶ Data of Eagle, Musselman, and Fleischman (1948). A single intramuscular injection of bacitracin at 36 units per kg caused the temporary disappearance of *T. pallidum* from the primary lesion. This dosage (1.2 mg per kg of a crude preparation) has been compared with the similarly effective total dose of penicillin G when given 3 times at 2 hour intervals to give a relative activity for bacitracin 5 per cent that of penicillin G. The difference in number of injections makes this an approximation only.



might fail to parallel their relative therapeutic activity in the established infection

Table 1 summarizes the available information with respect to the antisyphilitic activity of penicillins F, G, K, and X and of bacitracin, based on (1) their treponemicidal activity *in vitro* against the cultured Reiter strain (Eagle, 1946), (2) the dosages necessary to effect the disappearance of organisms from primary lesions in rabbits (Turner, Cumberland, Li, 1947, Eagle, Musselman, and Fleischman, 1948), (3) the dosages of each necessary to cure an established infection in rabbits when administered every 4 hours for 4 days and beginning 6 weeks after inoculation (Arnold, Boak, Carpenter, Chesney, Fleming, Gueft, Mahoney, and Rosahn, 1947), and (4) the dosages that suffice to abort an early infection in rabbits if administered every 4 hours for 24 doses, beginning 6 hours after intradermal inoculation with 60,000 organisms (Rake, Dunham, and Donovan, 1947)

TABLE 2  
*Material used in present study*

	LOT NO	SOURCE
F	123-BEL-4	Upjohn
G	46042605	Commercial Solvents
K	5/14/46	Pfizer
X	7/12/46	
	CA(81)IC	Lederle
	B-46	—
Bacitracin*	B-103	Ben Venue
	B-122	Ben Venue

\* Bacitracin is an antibacterial substance present in culture filtrates of *B. subtilis* (Tracy) Originally described by Johnson, Anker, and Meleney (1945), its treponemicidal action *in vitro* and *in vivo* has been described in another communication from this laboratory (Eagle, Musselman, and Fleischman, 1948)

There are here reported the results of an experiment to determine the abortive dose of these penicillins, as well as that of bacitracin, when administered intramuscularly once daily for 4 days, beginning 4 days after the intradermal inoculation of 2,000 organisms. The materials used are listed in table 2 and were obtained through the Antibiotics Study Section of the National Institute of Health. The courtesy of the pharmaceutical houses in making the compounds available for study is gratefully acknowledged.

The experimental data are detailed in table 3. The dosages of penicillins F, G, K, and X and of bacitracin that aborted infection in half the animals were 3.5, 0.3, 2.6, and 2.2 mg per kg, and 90 units per kg,<sup>1</sup> respectively. For penicillins F, G, K, and X, these represent relative gravimetric activities of 8, 100, 12, and 14, respectively, referred to that of penicillin G as 100. For a preparation of bacitracin assaying at 30 units per mg, the corresponding activity would be

TABLE 3

*The dosages of penicillins F, G, K, and X and of bacitracin necessary to abort early syphilitic infection in rabbits*

DOSAGE, MG/KG/ INJECTION	PENICILLINS								BACITRACIN		
	F		G		K		X		Dosage, units/ kg/inj	Non syphil itic	Syphil itic
	Nonsyph ilitic*	Syphil itic	Non syphil- itic	Syphil itic	Non syphil itic	Syphil itic	Non syphil itic	Syphil itic			
0 125			0	4							
0 25			3 (2)	3†			0	6			
0 5	0	2	5 (5)	1			1 (1)	5	18-20	0	4
1	0	6	6 (2)	0	0	4	3 (2)	3	36-39	1 (1)	7
2	3 (2)†	3	4	0	3 (3)	3	1	5	72-78	5 (2)	4
4	2 (2)	4	2	0	4 (4)	2	5 (3)	1	156	7 (5)	3
8			1	0	6 (3)	0	6	0	313	4 (4)	1
16					4	0			625	5	0
Dosage that aborted infection in half the ani- mals*	3 5± mg/kg		0 3 mg/kg		2 6 mg/kg		2 2 mg/kg		90 units/kg = 3 mg/kg of crude preparation containing 30 units/kg		
Relative activity per mg referred to that of peni- cillin G as 100	8		100		11 5		14		10§		

All animals were treated once daily for 4 days, beginning on the fourth day after their intradermal inoculation with 2,000 *T. pallidum*. Those animals in which a dark field positive lesion failed to develop at the site of inoculation were adjudged nonsyphilitic both on the basis of previous experience (Magnuson and Eagle, 1945, Eagle, Magnuson, and Fleischman, 1947) and the fact that in all but one of 41 animals tested, the popliteal lymph nodes proved to be noninfectious when inoculated into a normal animal 4 to 6 months after treatment. The number of animals so tested is indicated in parentheses in the body of the table. It is to be noted that these animals were deliberately selected from groups in which others had developed dark-field positive lesions, and in which asymptomatic infection might therefore have been expected were it a frequent occurrence.

\* After Reed and Muench (1938)

† One of these 3 rabbits developed asymptomatic infection demonstrated by the fact that a lymph node transferred to a normal rabbit 5 months after treatment proved infectious. This was the only asymptomatic infection in the entire group of 42 rabbits tested.

‡ The numbers in parentheses refer to those rabbits that did not develop a primary lesion, in which a lymph node transfer to a normal rabbit was carried out to confirm the fact that the animal had not been infected. All but one of the 42 rabbits so tested proved noninfectious.

§ Minimum value, applying to crude preparation assaying at 30 units per mg

10 per cent that of G, and for a preparation assaying at 90 units per mg (assuming the material can be purified to that extent), the percentage activity would be 30

## DISCUSSION

The results obtained in this and preceding studies with respect to the relative antisymphilitic activities of penicillins F, G, K, and X and of bacitracin are summarized in table 1. Their relative activities per mg, relative to that of penicillin

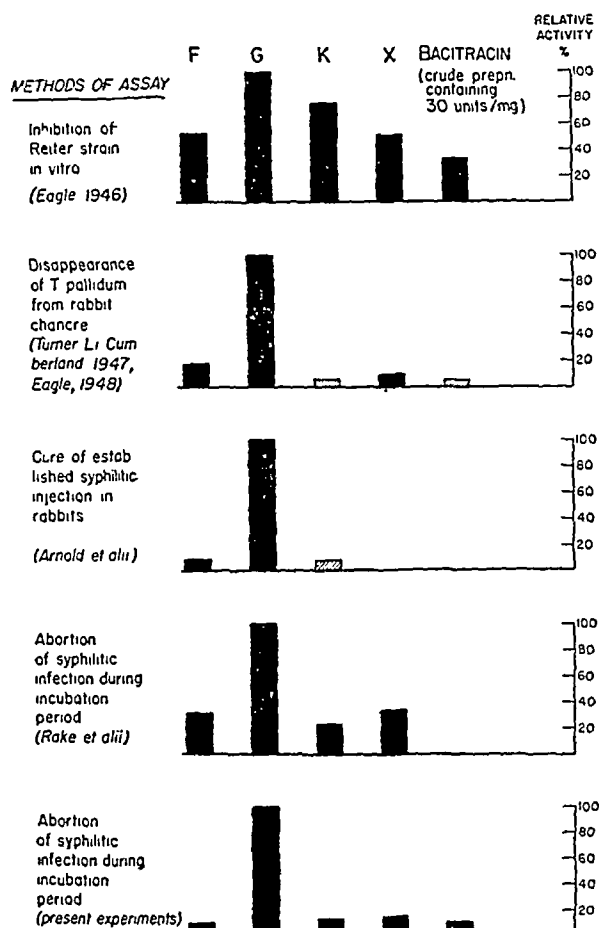


FIG 1 THE RELATIVE ACTIVITY OF PENICILLINS F, G, K, AND X AND OF BACITRACIN AGAINST *T. PALLIDUM* BY FIVE DIFFERENT METHODS OF ASSAY

Cross hatched blocks are results that are approximations only

G as 100, are shown graphically in figure 1. There are several aspects of these data that deserve emphasis.

(a) The absolute and relative activities of the various penicillins and of bacitracin varied widely according to the method of assay. Even two similar methods of assay, both based on the ability of these compounds to abort infection during the incubation period, gave discrepant results (cf. last two sections of table 1 and figure 1).

(b) Relative to penicillin G, penicillins F, K, and X as well as bacitracin were

significantly more active against the cultured Reiter strain *in vitro* than they were against the pathogenic *Treponema pallidum in vivo*

(c) By every method so far used, penicillin G was by far the most effectively antisyphilitic of the compounds tested. This is evident in figure 1. By the three methods of assay used to date in syphilitic rabbits, penicillin F was 7 to 31 per cent as active as G, K was <6 to 23 per cent, X was 9 to 34 per cent, and a crude preparation of bacitracin assaying at 30 units per mg was 4.6 to 10 per cent as active.

#### SUMMARY

Rabbits were inoculated intradermally with 2,000 *Treponema pallidum*, and treated 4 days later with intramuscular injections of the various penicillins or bacitracin, repeated once daily for 4 days. The doses of penicillins F, G, K, X and of bacitracin that aborted infection in half the animals were, respectively, 3.5, 0.3, 2.6, and 2.2 mg per kg, and 90 units per kg. Penicillins F, K, and X were therefore 8, 12, and 14 per cent as active per mg as penicillin G, and a crude preparation of bacitracin assaying at 30 units per mg was 10 per cent as active as penicillin G.

In table 1 and figure 1 the foregoing data have been compared with those obtained by other workers, using other techniques. It is apparent that not only the absolute but also the relative antisyphilitic activity of the several penicillins and of bacitracin varies widely according to the method of assay.

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# THE ACTION OF BACITRACIN AND SUBTILIN ON TREPONEMA PALLIDUM IN VITRO AND IN VIVO<sup>1</sup>

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Bacitracin is an antibacterial substance discovered by Johnson, Anker, and Meleney (1945) in culture filtrates of the so-called Tracy strain of *Bacillus subtilis*. The toxicity of the drug and its absorption and excretion in dogs have been described by Scudi and his coworkers (Scudi and Antopol, 1947, Scudi, Clift, and Krueger, 1947). The blood levels obtained after the intramuscular administration of bacitracin, its urinary excretion, and its renal clearance in rabbits and man have been reported in a previous communication from this laboratory (Eagle, Newman, Greif, Burkholder, and Goodman, 1947).

Subtilin is another agent produced by *B. subtilis*, described by Jansen and Hirschmann (1944). Its antibacterial activity *in vitro* and *in vivo* has been studied by Sallé and Jann (1945). It differs from bacitracin both in its antibacterial spectrum and in its chemical properties.

There have been no previous reports about the activity of these two antibiotics in the treatment of syphilis. As will be here reported, both were active *in vitro* against a cultured strain (Reiter) of *Treponema pallidum*, bacitracin being considerably more active than subtilin in this respect. *In vivo* also, bacitracin caused the disappearance of treponemata from testicular chancres in syphilitic rabbits and the prompt healing of the lesions. Subtilin, however, failed to effect permanent healing of the lesions in the largest doses used. The amount of bacitracin necessary to produce a permanent cure in rabbits is under present study, as is its activity in the treatment of the human disease.

## METHODS AND MATERIALS

*Bacitracin and subtilin.* The courtesy of the Western Regional Research Laboratories at Albany, California, and of Drs Eugene F Jansen and Howard D Lightbody in providing the subtilin used in these studies is gratefully acknowledged. Most of the bacitracin was prepared by the Ben Venue Laboratories at Bedford, Ohio.

The effective concentrations of bacitracin are expressed in the tables and figures in terms of the unit defined by Johnson, Anker, and Meleney, a unit of bacitracin being that "amount which when diluted 1:1024 in a series of two-fold dilutions in 2 cc of beef infusion broth, completely inhibits the growth of a stock strain of Group A hemolytic streptococcus when the inoculum used to seed the tubes is 0.1 cc of a 10<sup>-2</sup> dilution of an overnight culture in blood broth." The

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lots of bacitracin used in these experiments varied in activity from 18 to 40 units per mg, and there is reason to believe that the activity of the pure material may be at least twice the latter figure

The activity of subtilin is expressed in terms of milligrams, preliminary experiments in this laboratory with the Craig counter-current distribution apparatus (Craig, Hogeboom, Carpenter, and du Vigneaud, 1947) having indicated it to be a reasonably homogeneous material, at least as determined by its partition coefficient between water and butanol

The crystalline penicillin G used in these studies for comparison with bacitracin and subtilin was Lot no V31 supplied by the Squibb Institute for Medical Research of E R Squibb and Sons Their co-operation is gratefully acknowledged

*Inhibition of growth of treponemata in vitro* The Reiter strain of so called *T pallidum*<sup>2</sup> used in the *in vitro* experiments was grown on Brewer's thioglycolate medium, enriched with 10 per cent rabbit or human serum Sufficient additional agar was added (0.075 to 0.15 per cent) to thicken the medium and thus promote the formation of individual colonies in subculture

For the determination of the inhibitory effect of the antibiotics on growth, decreasing amounts of their solutions (0.8, 0.6, 0.4, 0.3, 0.2, etc.) were added to 9 ml of the thioglycolate-serum medium, the tubes were then inoculated with just 1,000 organisms from a growing, actively motile 48-hour culture, and the total volume was adjusted to 10 ml The number of colonies developing in each tube was counted after 7 to 14 days' incubation in an anaerobe jar at 37 C In the absence of antibiotic, from 2 to 4 colonies were usually obtained for each 10 organisms inoculated into the medium The control tube therefore contained on the order of 200 to 400 colonies, too many to count, and the experimental end point indicated in the tables was either that concentration which completely prevented growth, or that which permitted the development of only 20 colonies

*Inhibition of growth of Streptococcus pyogenes (C-203)* The concentrations of antibiotic necessary to prevent the growth of this organism were determined by a modified Rammelkamp-Kirby technique (Eagle, Newman, *et al*, 1947), using inhibition of hemolysis as the end point

*Rate of treponemicidal action in vitro* Bacitracin or subtilin was added in varying concentration to tubes of thioglycolate-serum medium at 37 C These were then inoculated with an actively growing 48-hour culture, to a final count of 10 million organisms per ml as determined by direct enumeration (Magnuson, Eagle, and Fleischman, 1948) At varying intervals thereafter (e.g., 3, 6, 12, 24, or 48 hours), aliquot portions were removed from the tubes, and the number of remaining organisms was determined by subculture in serial tenfold dilutions on thioglycolate medium In the absence of a specific method for the inactivation of the bacitracin or subtilin, dependence had to be placed on the serial dilutions to dilute the material to the point that it would not affect the growth in subculture of the surviving organisms

<sup>2</sup> The identification of these organisms as *T pallidum* is debatable They are probably saprophytic organisms that happened to be present in the syphilitic lesions when attempts were made at cultivation

In some experiments, the effect of the antibiotics on the number of organisms visible by dark-field examination, and on the proportion of those which were motile, was also determined

#### EXPERIMENTAL PROCEDURE AND RESULTS

*Concentration of bacitracin and subtilin necessary to inhibit the growth of T pallidum (Reiter) and of Streptococcus pyogenes (C-203) in vitro* Table 1 summarizes a number of experiments with four different lots of bacitracin and the cultured Reiter strain of so-called *T pallidum*. With inocula of 100 organisms per ml, 0.004 Johnson-Meleney units of bacitracin per ml completely inhibited the growth of the organisms, and an average of 0.002 units per ml permitted the

TABLE 1

*The inhibitory effects of bacitracin, subtilin, and penicillin G on the growth of T pallidum (Reiter) in vitro*

	INHIBITORY CONCENTRATIONS OF			
	Bacitracin*	Penicillin	Subtilin†	Penicillin
	units/ml	μg/ml	μg/ml	μg/ml
Complete inhibition‡				
Range	0.0024-0.0072	0.025-0.06	2, 3, 5	0.02, 0.03
Mean	0.0041	0.047		
Partial inhibition‡				
Range	0.0015-0.0036	0.015-0.35	1, 1, 1.95	0.015, 0.025
Mean	0.0023	0.027		
Conclusions as to relative activity	1 mg penicillin equivalent to an average of 90 units of bacitracin		1 mg penicillin equivalent to approximately 75-100 mg of subtilin	

\* Eleven experiments with 4 lots of bacitracin, varying in activity from 18 to 36 units per mg, each tested in parallel with penicillin G.

† One experiment with each of 2 lots tested in parallel with penicillin G.

‡ Complete inhibition, no colonies in 10-ml tube inoculated with total of 1,000 organisms, and yielding 200 to 400 colonies in a control tube containing no antibiotic. Partial inhibition, 20 colonies in a 10 ml tube similarly inoculated.

development of 20 colonies in a 10-ml tube. In simultaneous experiments with penicillin G, which was the most active of the four natural penicillins against this particular strain of treponema (Eagle, 1946), an average of 0.047 micrograms per ml prevented growth, and 0.027 micrograms per ml permitted the development of 20 colonies. One milligram of crystalline penicillin G, therefore, had an average activity equivalent to that of 90 units of bacitracin, i.e., penicillin G was three times as active as a crude preparation of bacitracin containing 30 units per mg.

As shown in the same table, subtilin was far less effective than either bacitracin or penicillin G. The totally inhibitory concentration was on the order of 2 to 4 micrograms per ml, and 1 to 2 micrograms per ml permitted the formation of

20 colonies Milligram for milligram, the two lots of subtilin tested were therefore approximately 1/70th to 1/100th as active as penicillin G against the Reiter strain, and 1/20th to 1/30th as active as a preparation of bacitracin assaying at 30 units per mg

The relative activities of bacitracin, subtilin, and penicillin against the C 203 strain of *Streptococcus pyogenes* (C-203) are shown in table 2. Measured by the concentrations necessary to inhibit growth, bacitracin was somewhat less active against this organism than it was against the Reiter strain of *T pallidum*, penicillin was several times more active, but subtilin was almost a hundred times more active. Subtilin was half as active as penicillin G against this strain of streptococcus *in vitro*, but only 1/70th to 1/100th as active as G against the Reiter strain of *T pallidum*.

TABLE 2

*The inhibitory effects of bacitracin, subtilin, and penicillin G on the growth of the C 203 strain of Streptococcus pyogenes in vitro*

	GROWTH INHIBITING CONCENTRATIONS AGAINST STREPTOCOCCUS PYOGENES OF			
	Bacitracin	Penicillin G	Subtilin	Penicillin G
	units/ml	μg/ml	μg/ml	μg/ml
Range	0.006-0.01*	0.0093-0.015	0.031, 0.037, 0.033†	0.015, 0.015, 0.014
Mean	0.008	0.013	0.034	0.015
Conclusions as to relative activity	1 mg penicillin equivalent to 600 units of bacitracin		1 mg penicillin equivalent to 2 mg of subtilin	

\* Eleven experiments with 4 lots of bacitracin. In each experiment penicillin G was tested simultaneously.

† Three experiments with 2 different lots of subtilin, each tested simultaneously with penicillin G.

*The rate of treponemacidal action of bacitracin and subtilin in vitro.* A number of experiments designed to establish the rate at which the treponemata were killed by bacitracin are summarized in table 3. One of those experiments is graphically illustrated in figure 1. With the much larger inocula used in these experiments (10 million per ml instead of 100), and over the shorter time period (24 to 48 hours instead of 7 to 14 days), it required larger concentrations of bacitracin to render the organisms nonviable than was the case in the experiments summarized in the previous section. In experiment 5 of that table a minimum concentration of 0.025 units per ml was necessary to effect a significant treponemacidal action, and a somewhat higher concentration (>0.025 but <0.1 unit per ml) was necessary in experiment 6. The smallest effective concentration of penicillin G similarly tested was 0.032 micrograms per ml (Eagle and Musselman, 1944, Eagle, 1946). By this criterion 1 mg of G therefore had an activity equivalent to that of 800 units of bacitracin, and it was some 25 times more effective than were crude preparations of bacitracin assaying at 30 units per mg.



Unlike the case of penicillin (Eagle and Musselman, 1944) there was no indication of a maximally effective level of bacitracin. The rate at which it killed the organisms increased with its concentration up to the highest level tested (64 units per ml, equivalent to 2 milligrams per ml of the particular preparation used).

TABLE 3

*The rate at which "T pallidum" (Reiter) was rendered nonviable by bacitracin in vitro*

EXP NO	UNITS/ML	TIME IN HOURS				TIME REQUIRED TO KILL 99.9 PER CENT OF ORGANISMS
		6	12	24	48	
		Proportion of organisms surviving (referred to viable organisms in original inoculum as 100)				
1	1 15	8 3		0 32	0 029	hr
	0 144	18		2 2	0 18	36
	0 0022	200		440	2,500	53
	0	—		680	2,000	∞
2-4	0 144	15, 17, 17	1, 2 2, 2 4	0 17, 0 016, 0 018	—	55, 39, 40
	0			415, 255, 255	—	—
5	4	8 8		<0 005	<0 005	<24
	0 2	66		0 38	0 02	35
	0 1	55		5 5	0 11	48
	0 05	88		5 5	1 3	>50
	0 025	100		22	9 4	>50
	0	150		1,150	6,650	—
6	64	0 03	—	—	—	6
	4	7 5	0 3	0 01	—	14
	1	9	1 5	0 018	0 015	18.5
	0 1	48 4	12 1	3	—	53±
	0 025		240	150	600	∞
	0 0125		180	480	3,000	∞
	0 0062	210	400	1,120	4,240	∞
	0	—	570	1,600	8,200	∞

**Conclusion.** Although a bacitracin concentration of 0.0062 units per ml caused a decreased net rate of multiplication over the first 48 hours, it required 0.025 units per ml to effect a net reduction in the number of viable organisms. Since the minimal effective concentration of penicillin G is 0.032 micrograms per ml (Eagle, 1946), 1 mg of penicillin was equivalent to approximately 800 units of bacitracin by this method of assay.

Unlike penicillin, there was no maximally effective concentration of bacitracin. The rate at which the organisms were killed increased with the concentration of antibiotic up to the highest level tested (64 units per ml).

At that concentration, 99.9 per cent of the organisms were killed in less than 6 hours, as compared with an average of 26 hours required at maximally effective concentrations of penicillin (Eagle and Musselman, 1948).

The progressive death of the organisms was reflected, not only in the decreasing number able to grow out as colonies in subculture, but also in the decreasing

number visible by dark-field examination and in their progressive loss of motility. One of several such experiments is illustrated in figure 2. A certain number of the organisms apparently undergo lysis, others are immobilized, and

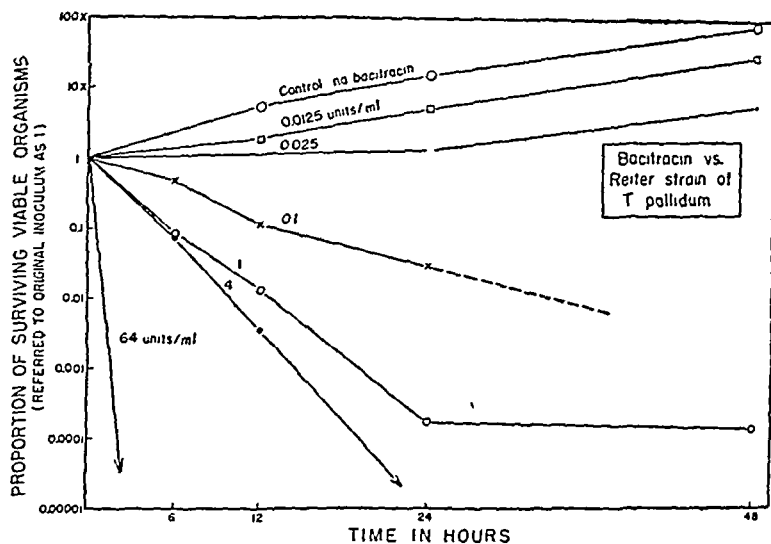


FIG. 1. THE EFFECT OF THE CONCENTRATION OF BACITRACIN ON THE RATE OF ITS TREPONEMICIDAL ACTION (CULTURED REITER STRAIN)  
Experiment 6 of table 3

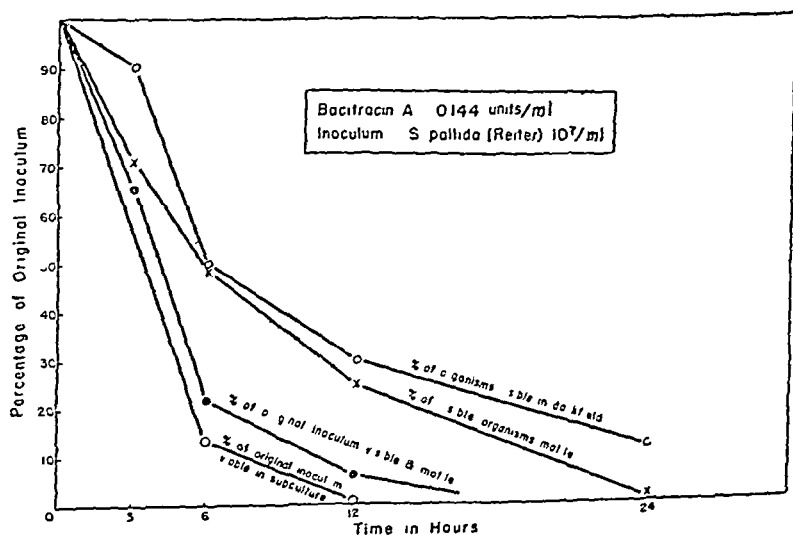


FIG. 2. THE TREPONEMICIDAL ACTION OF BACITRACIN IN VITRO (CULTURED REITER STRAIN)  
Lysis, immobilization, and loss of viability in subculture

the number able to grow out in subculture corresponds essentially to the number of visible and motile organisms, corrected for the fact that in control tubes only 20 to 40 per cent of the motile organisms inoculated develop into colonies on subculture.

Two experiments with subtilin are illustrated in table 4. The lowest concentration that decreased the net rate of multiplication was approximately 1 microgram, and the lowest concentration that had a demonstrable net bactericidal effect was 2 to 4 micrograms per ml. The latter concentrations were 64 to 128 times the similarly effective concentrations of penicillin G. As with bacitracin, and unlike penicillin, the treponemicidal activity of subtilin increased with its concentration up to the highest level studied (64 micrograms per ml). At that concentration, 99.9 per cent of the organisms were killed in  $5\frac{1}{2}$  hours, or 5 times faster than the maximal rate at which these organisms can be killed by penicillin G *in vitro*.

TABLE 4

*The rate at which "T pallidum" (Reiter) was rendered nonviable by subtilin in vitro*

EXP NO	SUBTILIN	TIME IN HOURS AT 37 C				TIME REQUIRED TO KILL 99.9 PER CENT OF ORGANISMS
		6	12	24	48	
		Proportion of organisms surviving (referred to viable organisms in original inoculum as 100)				
	micrograms/ml					hr
1	64	0.055		<0.005	<0.005	5
	8	5.5		0.016	<0.0005	10
	4	11		1.3	0.055	43
	2	44		9.4	6.6	>50
	1	120		—	260	—
	0	150		1,150	6,650	—
2	16	0.36	0			6
	8	7.5	0.015	0.006	0.0006	10
	4	27.2	—	0.63	—	33
	2	150	—	150	—	—
	1	330	570	1,230	3,030	—
	0.05		540	1,570	7,500	—
			570	1,600	8,200	

**Conclusion.** The lowest concentration of subtilin that effected a net decrease in the number of viable organisms within 48 hours was 2 to 4 micrograms per ml. Since penicillin G was similarly effective at a concentration of 0.032 micrograms per ml, it was 64 to 128 times more active per mg than the particular preparations of subtilin used in these experiments.

*The treponemicidal action of bacitracin and subtilin in vivo.* When bacitracin at dosages greater than 36 units per kg was injected intramuscularly into syphilitic rabbits with testicular chancres, motile treponemata usually disappeared from the lesions within 24 hours, and they were usually dark-field negative within 48 to 72 hours. As is shown in table 5, however, after single doses of as much as 2,560 units per kg, organisms would often reappear in the testis or epididymis days, weeks, or even months after treatment. In rabbits treated once daily for 4 days, similar relapses were noted in animals treated at doses up to 640 units per kg, but none in the seven animals treated at larger doses.

When penicillin was injected once daily for 4 days, treponemata reappeared

in the testes of two rabbits treated at 4,000 units per kg (24 mg per kg of penicillin G), but not in two treated at 8,000 units per kg. Gravimetrically, a crude preparation of bacitracin assaying at 30 units per mg was therefore on the order of one-tenth as active as penicillin in effecting the permanent healing of testicular chancres in rabbits.

The largest doses of subtilin so far used (10 mg per kg every 4 hours, repeated 4 times daily, and continued for 4 days) did not cause the permanent disappearance of organisms from the testicular chancres in any of the four rabbits tested.

TABLE 5

*The effect of treatment with bacitracin on the presence of T pallidum in testicular chancres of rabbits*

NO OF INJECTIONS	DOSAGE UNITS/KG PER INJECTION	CHANCER DID NOT BECOME DARK FIELD NEGATIVE AS RESULT OF TREATMENT	CHANCER BECAME TEMPORARILY DARK FIELD-NEGATIVE BUT ORGANISMS LATER REAPPEARED	CHANCER BECAME AND REMAINED DARK FIELD NEGATIVE THROUGHOUT PERIOD OF OBSERVATION (2 TO 4 MONTHS)	DOSAGE OF BACITRACIN AT WHICH CHANCER BECAME AND REMAINED DARK FIELD-NEGATIVE
One	36	1	0	0	Single injection of 5,120 units/kg
	72	0	2	0	
	144	0	2	1	
	288	0	1	1	
	576	0	0	4	
	640	2	0	0	
	1,154	0	1	3	
	1,280	0	2	1	
	2,560	0	3	5	
	5,120	0	0	4	
One injection daily for 4 days	36	3	0	0	1,150 units/kg repeated once daily for 4 days
	72	0	3	0	
	144	0	0	4	
	160	0	1	0	
	288	0	1	1	
	320	0	1	2	
	576	0	0	3	
	640	0	2	1	
	1,154	0	0	2	
	1,280	0	0	3	
	2,560	0	0	2	

This antibiotic was therefore even less effective against pathogenic *T pallidum* *in vivo* than was indicated by its direct bactericidal activity against the non pathogenic, cultured strain *in vitro*.

*Abortion of syphilitic infection.* If penicillin is administered a few hours to a few days after the inoculation of rabbits with *T pallidum*, a minute fraction of the dosage necessary to cure the established disease then suffices to kill the small inoculum and to abort the syphilitic infection (Magnuson and Eagle, 1945, Eagle, Magnuson, and Fleischman, 1947) Rake, Dunham, and Donovan (1947)

have developed a rapid method, based on this fact, for the assay of antisymphilitic agents. Since the criterion of failure is the development of a chancre at the site of inoculation, the assay can be completed within 2 to 3 months, as compared with the 12 months necessary for the ordinary therapeutic assay in established syphilitic infection, in which the criterion of cure is the noninfectiousness of a lymph node on transfer to normal rabbits 6 months after the completion of treatment.

The relative activity of bacitracin and penicillin G in preventing the development of syphilitic infection is shown in table 6 (Eagle and Fleischman, 1948). As there indicated, when animals were inoculated with 2,000 organisms and

TABLE 6

*The relative activity of bacitracin and penicillin G in the abortion of syphilitic infection in rabbits (after Eagle and Fleischman, 1948)*

PENICILLIN G				BACITRACIN			
Mg/kg per injection	Nonsyphilitic	Syphilitic	PD <sub>50</sub> mg/kg per injection*	Units/kg per injection	Nonsyphilitic	Syphilitic	PD <sub>50</sub> mg/kg per injection*
0.125	0	4	0.3	18	0	4	90
0.25	3 (2)	3		36-39	1 (1)	7	
0.5	5 (5)	1		72-78	5 (2)	4	
1	6 (2)	0		144-156	7 (5)	3	
2	4	0		313	4 (4)	1	
4	2	0		625	5	0	

Rabbits were inoculated intradermally with 2,000 organisms. Treatment with intramuscular injections of penicillin G or bacitracin was begun 4 days later, and continued once daily for 4 days. The failure of the animals to develop a syphilitic lesion at the site of inoculation was taken to indicate that the infection had been successfully aborted. The numbers in parentheses represent the number of animals in which this was confirmed by lymph node transfer into a normal rabbit 4 to 6 months after treatment. The others were not tested.

**Conclusion.** One mg of penicillin was as effective as were 300 units of bacitracin in the abortion of syphilitic infection in rabbits. By this method of assay, penicillin was therefore ten times as effective as a crude preparation of bacitracin assaying at 30 units per mg.

\* Dosage that protected 50 per cent of animals (calculated after Reed and Muench, 1938).

treated 4 days later by intramuscular injections repeated once daily for 4 days, a daily dose of 0.3 mg per kg of penicillin G sufficed to abort the infection in half the animals. The similarly effective dose of bacitracin was 90 units per kg. By this method of assay, 1 mg of penicillin G was therefore equivalent to 300 units of bacitracin, and penicillin was ten times as effective as a crude preparation of bacitracin assaying at 30 units per mg. The similarly abortive dose of subtilin was not determined.

Experiments are now in progress to determine the curative dose of bacitracin in established syphilitic infection of rabbits, using lymph node transfer 4 to 6 months after treatment as the criterion of cure, and to determine also whether it

exercises a synergistic action with penicillin Preliminary trials of bacitracin in the treatment of human patients are also in progress

### SUMMARY AND DISCUSSION

#### *Bacitracin*

Bacitracin has here been shown to have a definite treponemicidal action, both against the cultivated Reiter strain *in vitro* and pathogenic *Treponema pallidum in vivo* (cf table 7)

TABLE 7

*The relative activity of penicillin, subtilin, and bacitracin against Streptococcus pyogenes and treponemata summary of all experiments*

TEST ORGANISM	CRITERION OF ANTIBIOTIC ACTIVITY	ACTIVITY OF ANTIBIOTIC PER MG RELATIVE TO THAT OF PENICILLIN G	
		Bacitracin (im- pure prepn assaying at 30 units/mg)	Subtilin
<i>Streptococcus pyo- genes</i> , C-203	Inhibition of hemolysis <i>in vitro</i> (table 2)	% 5	% 50
Reiter strain <i>in vitro</i>	Inhibition of growth in 7 to 10 days (table 1)	33	1
	Direct bactericidal action in 24 to 48 hours (tables 3 and 4)	35	1 5
Treponemata	Permanent disappearance of organisms from primary lesion (table 5)	10	<1
	Abortion of syphilitic infection (table 6)	10	?
	Cure of experimental infection	?	1

**Conclusion** A crude preparation of bacitracin assaying at 30 units per mg was 5 per cent as active per mg as penicillin G against *Streptococcus pyogenes*, 35 per cent as active as G against the cultured Reiter treponema *in vitro*, and 10 per cent as active against the pathogenic *T pallidum*

Subtilin was 50 per cent as active per mg as penicillin G against the streptococcus, but only <1 to 1 5 per cent against the treponemata, either *in vitro* or *in vivo*

**Cultured Reiter strain in vitro** The drug caused the lysis and immobilization of the cultured organisms and a progressive loss of viability as judged by sub culture Approximately 0 004 units per ml completely inhibited growth, and 0 025 units per ml had a definite treponemicidal effect within 24 to 48 hours

Unlike the case of penicillin, there was no indication of a maximally effective concentration of bacitracin, instead, the rate of its treponemicidal action increased progressively up to the largest concentrations feasible to use experimentally in the absence of a simple method of inactivation Thus, at concentrations

of 0.1, 1, 4, and 64 units per ml, it required 53, 19, 14, and 6 hours to kill 99.9 per cent of the organisms.

*Pathogenic T pallidum in vivo* In syphilitic rabbits, small doses of bacitracin (36 units per kg) caused the rapid disappearance of the organisms from the primary lesion. The similarly effective doses of penicillin G are on the order of 0.11 mg per kg (Turner, Cumberland, and Li, 1947). Much larger doses were, however, necessary in order to effect the permanent healing of the chancre. The effective doses of bacitracin in this respect were 5,000 units per kg at one injection, or 1,150 units per kg repeated once daily for 4 days. The latter dosage (40 mg per kg of a crude preparation assaying at 30 units per mg) was approximately ten times the similarly effective dose of penicillin G.

In rabbits inoculated with 2,000 organisms 4 days previous to treatment with bacitracin, syphilitic infection could be aborted in half the animals by the administration of 90 units per kg of bacitracin, given once daily for 4 days. This dosage (3 mg per kg of a crude preparation assaying at 30 units per mg) was 10 times the similarly effective dose of penicillin G.

*Streptococcus pyogenes* Against the C-203 strain of  $\beta$ -hemolytic streptococcus *in vitro*, penicillin G was twenty times as active as a 30-unit-per-mg preparation of bacitracin.

#### *Subtilin*

Subtilin was more active than bacitracin against the C-203 strain of *Streptococcus pyogenes*, approaching the activity of penicillin in this respect. Against treponemata, however, whether the cultivated Reiter strain *in vitro* or the pathogenic organisms *in vivo*, it was far less active than either penicillin or bacitracin. The effective concentrations *in vitro* of 2 to 4 micrograms per ml were 75 to 100 times those of penicillin G, and the largest doses so far used, approximately 80 times the curative dose of penicillin similarly injected, failed to cause even the permanent disappearance of treponemata from a primary lesion in rabbits.

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# STUDIES ON A NEW OXALATE-DECOMPOSING BACTERIUM, *VIBRIO OXALITICUS*

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Only a few microorganisms are known to decompose oxalic acid or oxalate. Most of these are filamentous fungi, notably species of *Aspergillus* and *Penicillium*, which form oxalic acid under some conditions and decompose it under others (Wehmei, 1891, Bach and Fournier, 1935). Among bacteria the ability to decompose oxalate is very rare. Ayeis *et al* (1919) and Den Dooren de Jong (1928) tested over 125 strains isolated from a variety of sources and did not find a single one possessing this ability. Bassalik (1913) tested 90 species of bacteria and fungi and found only three strains of bacteria that could attack oxalate. Two of these decomposed oxalate very slowly in a synthetic medium, but the third, called *Bacillus extorquens*, decomposed oxalate rapidly and completely under the same conditions.

Bassalik made a rather thorough study of *Bacillus extorquens* and its action on oxalate. Since many of his observations can be directly compared with ours, it is worth while to summarize them briefly. *B. extorquens* was isolated originally from the excreta of an earthworm that had ingested plant material containing crystals of calcium oxalate, later it was shown to be present in forest and garden soils. The bacterium was enriched by inoculating the excreta or soil into a mineral medium containing ammonium oxalate as the only organic compound. Great difficulty was experienced in the isolation of pure cultures. Repeated attempts to use agar media failed, but the organism was finally isolated by the use of silica gel plates containing ammonium oxalate.

*B. extorquens* was a slightly bent, nonsporulating rod, averaging 1.5 by 3.0 microns in size and having a single polar flagellum. It formed a rose-red to blood-red pigment and, in a liquid oxalate medium, it grew characteristically as a film on the bottom and walls of the flask, leaving the liquid clear. Growth was poor on ordinary agar and gelatin media but was rapid and abundant on synthetic media containing one of the following compounds as a sole energy source: oxalate, glyoxalate, malonate, succinate, fumarate, maleate, oxamate, phenylacetate, formate, oxamide, methyl and ethyl alcohols, glycerol, sorbitol, mannitol, and glucose. Less vigorous growth was obtained with a variety of other compounds including glycolate and formaldehyde in low concentration. Both soluble and relatively insoluble oxalates, like the calcium and barium salts, were readily decomposed. The optimal concentration of soluble oxalates was between 0.1 and 0.3 per cent, although higher concentrations could be tolerated.

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provided the alkali resulting from the decomposition was neutralized. The decomposition of oxalate was shown to be an oxidation to carbon dioxide and water, the only other product being cell material. The rate of oxygen consumption on oxalate corresponds to  $Q_{O_2} = 32$  to 53 at 29 C. Finally it was shown that toluene-treated bacteria and even cell-free culture filtrates were capable of decomposing oxalate, a fact indicating the presence of a soluble enzyme.

Since the work of Bassalik very little has been done with oxalate-decomposing bacteria. Scholdei and Linstrom (1930) observed the disappearance of oxalate from a dilute solution, apparently as a result of bacterial action, but they were unable to isolate the causative organism. Barber and Gallimore (1940) showed that when an oxalate solution is inoculated with fecal material, oxalic acid rapidly disappears under both aerobic and anaerobic conditions. The process was evidently due to nonsporulating bacteria since it was prevented by pasteurizing the inoculum. No attempt was made to isolate or identify the bacteria.

The present paper deals with the isolation and characteristics of an aerobic soil bacterium, *Vibrio oxaliticus*, nov. spec., which is similar to *Bacillus enterquens* in its ability to decompose oxalate, but which differs from this organism in several important respects.

#### EXPERIMENTAL METHODS AND RESULTS

*Enrichment and isolation of V. oxaliticus.* For the enrichment of this organism a medium (No. 1) of the following composition in g per 100 ml was used: potassium oxalate hydrate, 0.1,  $(NH_4)_2SO_4$ , 0.05,  $K_2HPO_4$ , 0.05,  $MgSO_4 \cdot 7H_2O$ , 0.01,  $FeSO_4 \cdot 7H_2O$ , 0.002,  $CaSO_4 \cdot 2H_2O$ , 0.001, pH 7, made up with distilled water. A few drops of phenol red indicator were also added. The medium was inoculated with a small quantity of garden soil and incubated aerobically in a shallow layer at 28 C. Within 24 hours the medium became turbid and the pH rose to about 8.4. A permanganate titration showed that more than 85 per cent of the oxalate had been decomposed.

After one transfer in the same medium, the culture was streaked on an oxalate agar medium containing 0.1 per cent yeast extract. In 48 hours many small colonies of at least six different types appeared. Representative colonies were transferred by means of capillary pipettes into tubes of sterile liquid oxalate medium with yeast extract. In only two out of eight tubes was the oxalate decomposed, and microscopic examination showed that in both positive cultures at least two organisms were present, one a very small vibrio and the other a somewhat larger straight rod.

In order to facilitate the separation of these two organisms, which proved to be somewhat difficult, the plating medium was altered slightly by the addition of a small amount of sterile calcium chloride solution (2 ml 1/10 per 100 ml of medium 1). This resulted in the formation of a fine crystalline precipitate of calcium oxalate that made the agar slightly opaque. In such a medium the colonies of oxalate-decomposing bacteria can be easily recognized after a few days' incubation by the formation of a clear zone or halo about each as a result of the disappearance of the calcium oxalate crystals (figure 1). The dissolution

of the crystals in the immediate vicinity of the colonies can be observed microscopically even before the microscopic halos appear

By means of this plating technique, the vibrio was separated from the straight rod, and it was found that only the former was able to decompose oxalate. The available evidence indicates that the vibrio was the only oxalate-decomposing bacterium present in the enrichment cultures. The other five types of bacteria must have been living on organic materials synthesized by the vibrio.

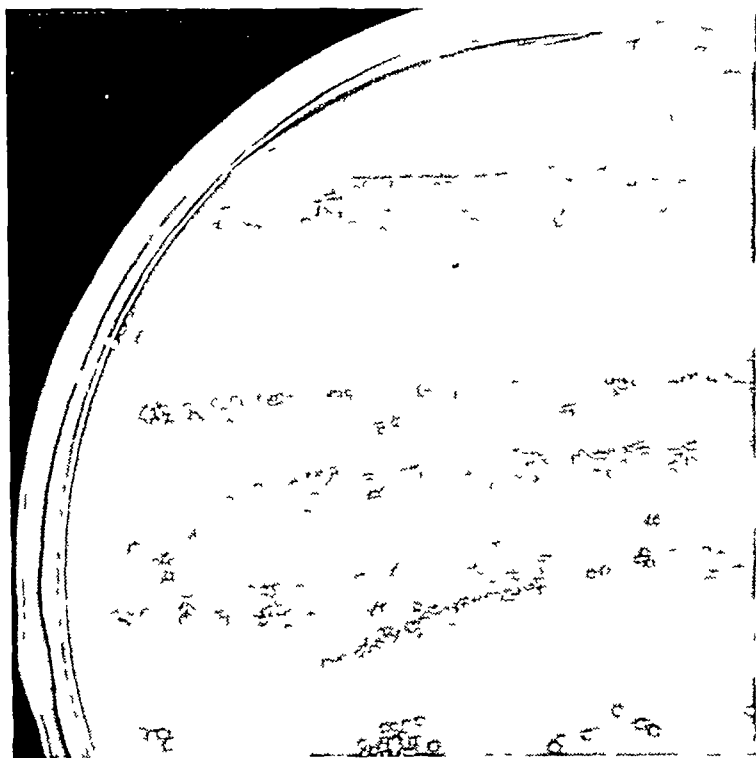


FIG. 1. CALCIUM OXALATE AGAR PLATE STREAKED FROM AN ENRICHMENT CULTURE AND INCUBATED 15 DAYS AT 28 C.

Growth was slow because all the oxalate was in the form of the calcium salt.

Four other strains of the vibrio were isolated from three soil samples obtained in Berkeley and one from the garden of the Massachusetts General Hospital in Boston. A number of other soil samples were examined for the presence of the vibrio by using them as inocula for enrichment cultures that were then examined microscopically. Positive results were obtained with all samples tested. Attempts to demonstrate the presence of oxalate-decomposing bacteria in air were unsuccessful.

Most of the experiments reported in this paper were done with strain 2.

*Morphology, staining, and cultural characteristics.* All five strains are similar in appearance and behavior. They are typical small vibrios, the average dimen-

sions being  $0.4 \times 1.3$  microns (figure 2). The cells in young cultures are actively motile by means of a single polar flagellum, 6 to 8 times the length of the cell body. No capsules or spores were observed. The cells were gram-negative at all times.

The organism grows on nutrient agar to form small, pin-point colonies in about 48 hours that were moist, raised, and had entire edges. The colonies grow slowly on further incubation and reach a maximal diameter of 1.5 mm in about 6 days. Colonies were never pigmented. Moderate growth was obtained in nutrient broth, after 24 hours it was mostly confined to a thin surface film, and after another 24 to 48 hours a slight general turbidity developed. The organism does not reduce nitrate, and does not form indole or hydrogen sulfide.



FIG. 2. *V. OXALITICUS*, STRAIN 1, FROM 2-DAY-OLD CULTURE ON AN OVALATE MEDIUM. Gentian violet stain, 1,000  $\times$ .

On calcium ovalate agar containing 0.1 per cent yeast extract growth is rapid, but the colonies remain small because of the limited quantity of available nutrients. The colonies are similar in size and appearance to those on nutrient agar. It should be noted that ovalate-containing media always tend to become alkaline as the anion is decomposed. When phenol red is present, the color changes from yellow to red. In a liquid ovalate medium with or without yeast extract, a slight surface film forms first, and later a general turbidity develops. Growth is more rapid in shaken than in stationary cultures, probably because of better aeration. In a culture incubated on a shaker, 0.1 per cent potassium ovalate is decomposed almost completely within 24 hours at 28  $^{\circ}$ C.

*Substrates utilized for growth.* A number of organic compounds were tested

growth substrates by substituting them for oxalate in medium 1 with and without the addition of 0.05 per cent Difco yeast extract. Utilization of the substrate was judged by an increased turbidity over the control with no added substrate. It was found that in the presence of yeast extract the only compounds that support growth within 3 to 4 days are oxalate, formate, and pyruvate. In this period of incubation the organism is unable to utilize acetate, butyrate, citrate, lactate, malate, malonate, succinate, tartrate, or glucose. However, acetate at least does support abundant growth when the incubation period is further extended. At the end of 3 to 4 days there is only a slight effect of acetate, but growth continues slowly and reaches a maximum on the twelfth day. This indicates that an adaptation is required for acetate utilization. The lag period can be considerably reduced by transferring the bacteria several times in acetate media. When the various compounds were tested as growth substrates in the absence of yeast extract, the results were qualitatively the same except with formate, which does not support growth when it is the sole carbon source.

*Taxonomy* The morphology of the oxalate-decomposing organism places it in the genus *Vibrio* as defined in *Bergey's Manual* (1939). It could not be identified with any species described therein. There is a generic similarity between our organism and Bassalik's *B. extorquens*, but they differ in many details. For example, *B. extorquens* is a relatively large vibrio that forms a red pigment and metabolizes a great variety of organic compounds in a mineral medium. Our organism, on the contrary, is very small, does not form any pigment, and only grows well with oxalate and pyruvate in a mineral medium. In view of these obvious differences, we must regard our organism as a new species for which we propose the name, *Vibrio oxaliticus*.

*Quantitative experiments on growth with various substrates* It has already been pointed out that *V. oxaliticus* can grow in a mineral medium with oxalate, acetate, or pyruvate as the sole energy source. Yeast extract also supports growth. The amount of growth depends upon the substrate and its concentration. The data presented in table 1 show that, at a given concentration, pyruvate supports the heaviest growth, acetate is next, yeast extract third, and oxalate gives the poorest growth. Pyruvate is more than ten times as effective as oxalate. This is no doubt a reflection of the different energy contents of the two compounds. It should be noted that the rate of growth is not necessarily parallel with the cell yield. Acetate is second only to pyruvate in efficiency of utilization for cell synthesis, but it is metabolized much more slowly than are the other compounds studied.

*V. oxaliticus* prefers relatively dilute media. With oxalate, acetate, and pyruvate the heaviest growth is obtained at or below 0.3 per cent. With yeast extract, growth continues to increase with concentration up to about 2 per cent and then falls off at higher concentrations. The low concentration optimum for oxalate might be explained by a need for calcium, which is made progressively more unavailable as the oxalate is increased. There is no obvious explanation, however, for the similarly low optima with acetate and pyruvate.

Several experiments were done in order to find out what constituent of the

yeast extract is used as a growth substrate. These experiments have not led to a definite conclusion, but they indicate that the active constituent is not an amino acid or other nitrogenous compound. This may be deduced from the fact that no ammonia is formed from yeast extract, and also from the observation that growth is not improved by the addition of casein hydrolyzate, which consists largely of amino acids. Acetate, formate, and pyruvate are not present in yeast

TABLE 1  
*Influence of substrate concentration on growth\**

SUBSTRATE CONC %	MAXIMAL TURBIDITY†			
	Difco yeast extract	Potassium oxalate	Sodium acetate	Sodium pyruvate
0.1	21	9	83.5	118
0.2	33	21	102	187
0.3	51	25	78	235
0.4	65	24	59	235
0.6	84			168

\* Medium 1 with the oxalate replaced by the indicated amounts of the various substrates

†  $(2 - \log G) \times 100$  Determined with an Evelyn photocolormeter

TABLE 2  
*Influence of formate and yeast autolysate on growth\**

YEAST EXTRACT g/100 ml	MAXIMAL TURBIDITY†		
	- Formate	+ Formate‡	$\Delta$
0	0	0	0
0.05	11	35	24
0.1	21	56	35
0.2	33	88	55
0.4	65	138	73
0.8	121	143	22
1.6	184	184	0

\* Medium 1 was used without oxalate and with the indicated additions

†  $(2 - \log G) \times 100$

‡ 100 mg sodium formate per 100 ml

extract in sufficient quantities to account for more than a minute fraction of its activity.

The utilization of formate in the presence but not in the absence of yeast extract has already been noted. This effect was studied in more detail by determining the maximal growth obtained with various concentrations of yeast extract with and without formate. The data are given in table 2. It can be seen that growth on yeast extract is greatly increased by the addition of formate. Furthermore the effect of a given amount of formate increases with the concentration

of yeast extract up to 0.4 per cent and then falls off to zero at much higher concentrations. In a second experiment the yeast extract concentration was kept constant at two levels, whereas the formate was varied over a wide range. The data presented in table 3 show that, with 0.1 per cent yeast extract, growth increases with formate concentration up to 0.2 per cent and then declines. With 0.4 per cent yeast extract, maximal growth is obtained when 0.1 per cent formate is present, growth decreases progressively as the concentration is raised further.

These results are consistent with the view that formate is an incomplete substrate that serves as an energy source but does not provide all the compounds essential for cell synthesis. When yeast extract is provided as a source of essential metabolites, the energy derived from the oxidation of formate can be used with an efficiency that increases with the supply of other compounds. However, the addition of an excess of yeast extract undoubtedly provides other energy

TABLE 3

*Influence of formate concentration on growth with different amounts of yeast extract\**

SODIUM FORMATE  g/100 ml	MAXIMAL TURBIDITY†	
	0.1% yeast extract	0.4% yeast extract
0	21	67
0.1	49	108
0.2	81	78
0.3	37	69
0.5	25	49
0.8	7	25

\* Medium 1 without oxalate and with the indicated amounts of formate and Difco yeast extract

†  $(2 - \log G) \times 100$

sources, which make formate superfluous. The diminished growth at higher formate concentrations may be due to formate toxicity.

As a supplement to formate, yeast extract could not be replaced by any of the known water-soluble growth factors, either alone or in combination.

*Respiration of cell suspensions.* The ability of cells grown on a medium containing 0.2 per cent potassium oxalate and 0.1 per cent yeast extract to oxidize a number of organic compounds was tested manometrically using alkali to absorb carbon dioxide. Only three compounds caused an oxygen uptake greater than that of the control without substrate, namely, oxalate, formate, and pyruvate. The following compounds were not oxidized at a measurable rate: oxamate, oxamide, oxalurate, allantoin, allantoate, acetate, succinate, and hydrogen.

The respiratory rates on oxalate, formate, and pyruvate were determined using cells grown on each of these three substrates. The data given in table 4 show that the enzymes involved in the utilization of oxalate and formate are formed to about the same extent on all three growth substrates. The pyruvate enzyme,

also, is always formed, but its activity varies considerably with the substrate. It is curious that the highest activity toward pyruvate was exhibited by cells grown on formate.

The absolute oxygen uptakes and respiratory quotients observed in the same experiment are given in table 5. The values obtained with the substrates have not been corrected for the endogenous respiration. The data indicate that oxalate and formate are almost completely oxidized to carbon dioxide. With pyruvate the respiratory quotient is close to the theoretical value of 1.2, but the

TABLE 4  
*Respiratory rates for cells grown on different substrates*

GROWTH SUBSTRATE	Q <sub>O<sub>2</sub></sub> (N)			
	Respiration substrate (0.1 ml $\mu$ /10 per 2 ml)			
	None	Oxalate	Formate	Pyruvate
Yeast extract + oxalate*	81.4	225	235	135
Yeast extract + formate*	148	270	310	630
Yeast extract + pyruvate*	67	290	270	430

\* Medium 1 with 0.1 per cent Difco yeast extract and 0.2 per cent of potassium oxalate, sodium formate, or sodium pyruvate.

TABLE 5  
*Oxygen uptake and respiratory quotients*

GROWTH SUBSTRATE	RESPIRATORY SUBSTRATE (10 $\mu$ M)							
	None		Oxalate		Formate		Pyruvate	
	O <sub>2</sub> uptake $\mu$ M	R Q	O <sub>2</sub> uptake $\mu$ M	R Q	O <sub>2</sub> uptake $\mu$ M	R Q	O <sub>2</sub> uptake $\mu$ M	R Q
Yeast extract + oxalate	1.60	1.02	4.43	3.39	4.61	1.81	(3.32*)	1.29
Yeast extract + formate	2.87	1.10	5.34	—	6.12	1.75	12.4	1.33
Yeast extract + pyruvate	1.64	1.04	7.17	3.02	6.61	1.60	10.5	1.37
Theoretical for complete oxidation			5.0	4.0	5.0	2.0	25.0	1.2

\* The substrate decomposition was incomplete.

oxygen uptake and carbon dioxide production are only about half those required for complete oxidation. The other half of the pyruvate carbon is probably assimilated. The results are entirely consistent with the behavior of growing cultures. We have seen that the quantity of cells obtained with pyruvate is much greater than with oxalate or formate.

#### DISCUSSION

Our observations, considered in conjunction with those of Bassahk, indicate that oxalate-decomposing bacteria are commonly present in soil. This is not unexpected in view of the fact that substantial quantities of oxalate are being



continually added to soil in plant residues. The presence of these bacteria can be demonstrated very easily by the use of an enrichment medium containing oxalate as the only energy source. The isolation of the bacteria in pure culture is somewhat more difficult but is greatly facilitated by the use of a solid medium containing crystals of calcium oxalate.

*Vibrio oxaliticus*, which we have isolated from California and Boston soils, is probably closely related to Bassalik's *Bacillus extorquens*, isolated in Switzerland. In spite of its name, the latter organism is a typical *Vibrio* and it should be called *Vibrio extorquens* to fit into modern systems of bacterial taxonomy. The details of the morphology and physiology of the two organisms are, however, sufficiently different so that there can be no doubt that they represent distinct species.

Oxalate and formate are very simple substrates. The removal of two electrons from either compound will result in its conversion to carbon dioxide. This leads to the idea that the metabolism of *V. oxaliticus* may be similar to that of the autotrophic bacteria in the sense that energy is obtained by the oxidation of a simple compound, whereas the cell materials are built up from carbon dioxide. There is no a priori reason why energy for the reduction of carbon dioxide could not be obtained from the oxidation of oxalate just as well as from the oxidation of hydrogen or nitrite, for example. There is no definite evidence at present, however, in favor of this view. We do not know whether in fact *V. oxaliticus* is able to utilize carbon dioxide, although this could be determined by experiments with carbon isotopes. There is one bit of evidence against the foregoing view, namely, the fact that the organism cannot grow with formate alone. If carbon dioxide can be reduced with oxalate as an energy source, one would expect the same to occur with formate. Since formate cannot replace oxalate completely, it is quite possible that oxalate serves as a starting point for synthetic reactions as well as an energy source. In any event it is clear that the metabolism of simple substrates like oxalate presents many intriguing and fundamental questions for which there are as yet no answers.

#### SUMMARY

The isolation and characteristics of a new oxalate-decomposing bacterium, *Vibrio oxaliticus*, are described. The organism is able to grow on oxalate, pyruvate, and acetate in an otherwise mineral medium. Its growth is also increased by the oxidation of formate in a medium containing yeast extract. The oxalate and formate enzymes are constitutive, whereas the activity of the pyruvate enzyme system varies with the growth substrate. Oxalate and formate are almost completely oxidized to carbon dioxide, pyruvate is about half-oxidized and half-used for cell synthesis.

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# RESISTIVITY OF ESCHERICHIA COLI TO ULTRAVIOLET ENERGY ( $\lambda 2537$ ) AS AFFECTED BY IRRADIATION OF PRECEDING CULTURES

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The question arises from time to time as to the possible effect sublethal dosages of germicidal energy may have upon the resistivity to ultraviolet energy of subsequent cultures of a given organism

To investigate the possibility of such a change in an organism, we chose *Escherichia coli*, S A Waksman strain 9637. A 24-hour culture was used in each test, 0.005 ml broth culture being taken with 4,000 ml 0.87 per cent saline, and 4 ml of sterile broth were added for nutriment. The diluted *E. coli* suspension was placed in a large open-top petri dish to a depth of 1 inch. A source of germicidal energy ( $\lambda 2537$ ) with a mechanical shutter was placed above the dish at a point that provided an incident intensity of ultraviolet energy ( $\lambda 2537$ ) of 50 microwatts per sq cm. The remainder of the *E. coli* suspension was retained as an unirradiated control. It has been shown that the ultraviolet energy of  $\lambda 2537$  is in the spectral region of maximal germicidal effectiveness (Luckiesh, 1946).

The test solution was irradiated for 0.4 minutes. This exposure produced a dosage of 20 *Et* (intensity *E* of energy of  $\lambda 2537$  in microwatts per cm<sup>2</sup> times exposure-time *t* in minutes) at 50 mw per cm<sup>2</sup>. A sample of 1 ml was then taken from both the test and control suspensions and a poured plate of each was made immediately. The test solution was then given a second exposure of 0.4 minutes, making the total accumulated dosage 40 *Et*, and another poured plate was made from each of the test and control solutions. This process continued, methodically increasing the exposures until the final dosage was 250 *Et*. On observing the culture plates after 24 hours of incubation, we noted that no colonies survived in test samples receiving 120 *Et* or more.

A colony developing from a test sample surviving the highest sublethal dosage (100 *Et*) of germicidal energy was then used to inoculate a subculture called strain UV-1. A parallel subculture was made from a colony developing from an unirradiated sample. This was called strain 1. Strain UV-1 and strain 1 were incubated under identical conditions. Tests of resistivity to ultraviolet energy of  $\lambda 2537$  were made on these two strains by the method already described. Observation of the results of the resistivity test on strain UV-1 indicated that no organisms survived a dosage of 200 *Et*, but a few survived 180 *Et*. A colony surviving this highest sublethal dosage was used to inoculate strain UV-2. The previously unirradiated strain 1 showed no colonies appearing in cultures receiving a dosage of 100 *Et* and above.

This irradiation and subculturing process was repeated 5 times, each time the subculture for the next resistivity test on the irradiated strain being inoculated

from a colony developing from an organism surviving the highest sublethal dosage of germicidal energy. The subculture for the control strain was made in each test from an unirradiated culture sample. The average resistivity curves of irradiated and unirradiated strains are shown in figure 1. The resistivity curves obtained in these five series of tests did not differ markedly. This fact is also

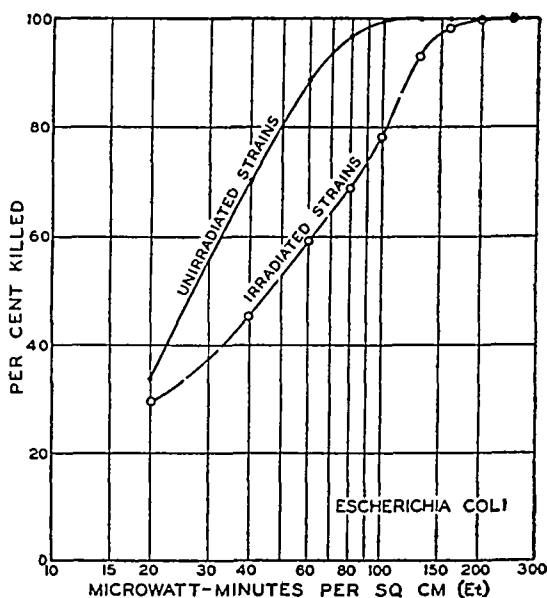


FIG 1 AVERAGE DEATH RATES OF IRRADIATED AND UNIRRADIATED STRAINS OF *E. coli* AFTER VARIOUS DOSAGES  $Et$  OF ULTRAVIOLET ENERGY OF  $\lambda 2537$

Dosages that result in a high percentage killed are about twice as great for the irradiated strains as for the unirradiated strains

TABLE 1

*Relative dosages of germicidal energy required for 100 per cent kill of irradiated and unirradiated strains of Escherichia coli*

STRAIN NO	IRRADIATED	STRAIN NO	PREVIOUSLY UNIRRADIATED
UV-1	200 $Et$	1	100 $Et$
UV-2	180	2	100
UV-3	200	3	120
UV-4	200	4	120
UV-5	200	5	100
UV 6	190	6	120

indicated by the data in table 1 in which the minimum dosage is shown for 100 per cent kill for the strains designated "irradiated" strains UV-1 to UV-5 and "unirradiated" strains 1 to 5. Values above 99.5 per cent kill were taken as 100 per cent for simplicity of interpretation of the data. It is seen that the apparent resistivity of the "irradiated" strains is, on the average, nearly twice that of the "previously unirradiated" strains.

After observation of the increase in resistivity of the irradiated strains UV-1 through UV-5 as compared with the previously unirradiated strains 1 through 5, experiments were made to determine whether by repeated subculturing without additional exposure to germicidal energy the irradiated strain would return to its original resistivity or maintain its apparent change. Both strains were subcultured under identical conditions through 6 successive transfers over a period of 20 days, after which a germicidal irradiation test was run on these strains, herein identified as subculture 6 in table 1.

Table 2 presents the relative resistivities, as indicated by the percentage killed by a dosage of 100 *Et*, for the irradiated and previously unirradiated strains of *E. coli*.

The germicidal energy produced a more resistant form in the irradiated strain. Colonies growing on Levine's eosine methylene blue agar showed less typical greenish metallic sheen than did the unirradiated strain. After storage of cul-

TABLE 2

*The relative resistivities of the irradiated and unirradiated strains of Escherichia coli indicated by the percentage of kill with a dosage of 100 Et*

IRRADIATED STRAINS		PREVIOUSLY UNIRRADIATED STRAINS	
Strain no	Per cent kill	Strain no	Per cent kill
UV-1	69	1	100
UV 2	85	2	100
UV-3	83	3	99
UV-4	86	4	99
UV 5	79	5	99 5
UV-6	67	6	99

tures on E M B of the irradiated strain for 5 days at room temperature, the colonies became mucoid and purple in color, losing all traces of metallic greenish sheen, whereas E M B cultures of the previously unirradiated strain maintained typical colony appearance. Nine biochemical reactions were investigated. These included the methyl red test, the production of indole, and the fermentation of lactose, maltose, xylose, mannitol, glucose, sucrose, and dulcitol. The only difference noted between the two strains was that the irradiated strain did not ferment dulcitol, whereas the unirradiated strain did, producing acid and gas.

#### DISCUSSION AND SUMMARY

Irradiated cultures of *Escherichia coli* developed greater resistivity to measured sublethal dosages of germicidal energy than unirradiated cultures. The greatest increase observed in the resistivity of the irradiated strains of *E. coli* was approximately 100 per cent. Stated in another manner, the minimum dosage *Et* necessary to effect a 100 per cent kill of the irradiated strains was about twice the minimum dosage necessary to effect a 100 per cent kill of the unirradiated strains.

The theory could be advanced that the increased ultraviolet resistivities of *E. coli* strains UV-1 to UV-6, inclusive, compared with previously unirradiated

strains, 1 to 6, as shown in table 1, were not caused by ultraviolet irradiation but by inoculating strain UV-1 with an organism of much higher resistivity than that of the average organisms in the S A Waksman strain 9637. If this were true, one might expect the irradiated strains, UV-2 through UV-6, to follow the same pattern in resistivity. The authors do not believe that strain UV-1 was started from an abnormally highly resistant organism for, as shown in table 1, no organisms in strains 1 through 6 survived a dosage over 120 Et. We, therefore, feel that no more highly resistant organisms existed in the original strain and that the increased resistance of strains UV-1 to UV-6, inclusive, was due to irradiation with ultraviolet energy in the spectral region of  $\lambda 2537$ .

It would be of considerable interest to investigate the possible variation of an organism from strains receiving lesser sublethal dosages than those used by the authors. Another variation would be to study strains receiving the same dosages as those used in the present investigation but at much lower levels of energy intensity. Such treatment may produce significant differences in the microorganisms to affect their metabolism and pathogenicity.

We acknowledge the technical skill of E T Leppelmeier in prosecuting these investigations.

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# ON THE ANTIGENIC STRUCTURE OF THE BACTERIAL SPORE

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Defalle (1902), Mellon and Anderson (1919), Howie and Cruickshank (1940), Lamanna (1940*a, b*), and Bekker (1944) have reported that bacterial spores are capable of inducing the formation of specific antibodies and give no evidence in agglutinin-absorption experiments of possessing antigens related to vegetative cells. In contrast to these findings is the work of Krauskopf and McCoy (1937), who, using alkali-treated spores of *Bacillus niger* to prepare antiserum, found a serological relationship of spores to vegetative cells. They concluded that spores did not possess antigenic factors not found in vegetative cells. The present series of experiments has been directed toward a more complete understanding of the spore in its antigenic relation to its mother cell and toward the reconciliation of the data of Krauskopf and McCoy with the experience of other investigators.

Cross agglutination between spores from various species, which would require a complex antigenic structure for the spore, has been denied by Bekker (1944). On the other hand, Defalle (1902) and Lamanna (1940*b*) have reported some evidence suggestive of such cross reactions, a finding which is developed further in this paper.

## MATERIALS AND METHODS

The strains used were *Bacillus cereus* C3, *Bacillus subtilis*, Ford strain S8, *Bacillus vulgatus*, or Marburg strain of *Bacillus subtilis* C4, *Bacillus agri* 13, *Bacillus brevis*, Ba8 (4), a strain kindly supplied by Dr. K. L. Burdon, *Bacillus* sp. B40 (otherwise identified as *B. brevis* by Smith, Gordon, and Clark, 1946), *Bacillus sphaericus* var. *fusiformis* A20, identified by Smith, Gordon, and Clark. Unless stated otherwise, the identifications are our own.

Antisera to spores, vegetative cells and H and O antigens were produced in different rabbits by giving a series of 5 to 7 intravenous injections of 0.5 to 1 ml of the material containing 50 to 200 million cells. Vegetative cells were grown overnight fresh for each injection. H antigens were prepared by the addition of 0.2 per cent formalin to a suspension of washed vegetative cells. O antigens were prepared by boiling a suspension of washed vegetative cells for 2½ hours. Spore suspensions were obtained by seeding the cultures on asparaginate agar (Howie and Cruickshank, 1940), or beef extract agar (Lamanna, 1940*a, b*), and incubating for about 2 weeks at 34, 37, or 45 C depending upon the strain. Sporulation was in general more rapid and complete on asparaginate agar, although there was some variation among the species in this regard. Spores were washed thoroughly with saline and stored at 0 C. The same suspension was used for injections, agglutination trials, and absorptions.

Precipitinogens were prepared by either the formamide extraction method of Fuller (1938) or the acid extraction method (Lamanna, 1942), and sometimes by both methods. The precipitin reaction was set up as a qualitative ring test. The antigen was layered over the antiserum, and the appearance of a ring of flocculus by the end of an hour at 37 C, or overnight at 0 C, was considered positive. Proper control tubes were included.

Spore agglutinations were read with ease when the shaking method of Lamanna (1940a) was employed.

Technical difficulties encountered in working with this genus included spontaneous clumping of vegetative cells, *B. cereus* and *B. brevis* were the worst offenders in this respect. Though young cultures grown on nutrient agar were used frequently with success, occasionally they would unaccountably clump. The clumps were allowed to settle to the bottom of the suspension before the preparation was used.

The possibility that there may be vegetative cells, or antigenic remnants of them, in the spore suspensions is undeniable. In fact, bacillary forms could sometimes be seen microscopically. Conclusions as to spore-vegetative cell cross reactions were, therefore, based largely on experiments in which spores were used only as agglutinative antigens, and the antisera were prepared against living fresh vegetative cell material. There is little danger of accidentally including spores in properly prepared vegetative cell vaccine.

#### EXPERIMENTAL RESULTS AND DISCUSSION

After the first series, or the first several series of injections, rabbit antisera to vegetative cells and to H and O antigens contained no demonstrable antibody against the homologous spore antigens (table 1). Prolonged immunization with living vegetative cell vaccine was then undertaken and found to produce an antibody that would agglutinate the spores, although in low titer. In general, the highest antispore titers accompanied the highest antivegetative-cell titers (table 2). These results suggest that there is present in the vegetative cell, in very small amounts or otherwise obscured, an antigen that also occurs in the spores.

Vegetative cells of *B. niger* were found by Krauskopf and McCoy to induce antibody against spores. To test their observation in reverse, these authors used a spore antiserum produced by injection of potassium-hydroxide-treated spores. This antiserum contained vegetative cell antibody and no specific spore antibody. They concluded from agglutination and agglutinin-absorption experiments that there were only antigenic factors common to the two stages. As a check on this work, we tried alkali treatment with spores of C3.

Potassium hydroxide in a final concentration of 5 per cent was left in contact with the spores until they would take a carbol fuchsin stain without heating. This required about 6 hours, a considerably longer time than that used by Krauskopf and McCoy. The treated spores were washed three times with 0.85 per cent saline solution and used for injection into one rabbit. Cross agglutination and reciprocal cross-agglutinin-absorption experiments were performed with



antiserum from this rabbit and with an antiserum to vegetative cells of the same strain. The results obtained are shown in figure 1. The entire block shows the titer of the unabsorbed serum. The solid fraction of the blocks indicates the titer after absorption.

TABLE 1

*Agglutination tests with vegetative, O, H, and spore antigens against vegetative, O, and H antisera*

Antigen Titer*	ANTISERUM															
	C3 V		C3 H			C3 O			C4-V		C4-H			C4-O		
	C3-V	C3-S	C3 H	C3-O	C3 S	C3 H	C3 O	C3-S	C4-V	C4-S	C4-H	C4-O	C4-S	C4-H	C4-O	C4-S
	256	0	4096	0	0	0	512	0	1024	0	256	64	0	128	4096	0

V, vegetative cells

S, spores

\* Highest dilution of serum in which agglutination occurred

TABLE 2

*Appearance of spore antibody in vegetative cell antisera as a result of long continued immunization*

VEGETATIVE CELL ANTISERUM	NUMBER OF INJECTION SERIES AND DATE OF BLEEDING*	HOMOLOGOUS VEGE TATIVE CELL TITER	HOMOLOGOUS SPORE TITER
Rabbit no 13 C3-V	1—Sept 11, 1946	256	0
	2—Sept 27, 1946	512	8
	3—Oct 30, 1946	8,192	0
	4—Feb 2, 1947	8,192	256
	5—April 27, 1947	8,192	128
	6—July 14, 1947	8,192	256
Rabbit no 19 C3-V	1—Sept 27, 1946	256	0
	2—Oct 30, 1946	8,192	0
	3—July 14, 1947	8,192	128
Rabbit no 16 S8 V	1—Sept 21, 1946	16	0
	2—Oct 30, 1946	256	4
	3—Feb 3, 1947	1,024	64
	4—April 25, 1947	64	8
	5—July 14, 1947	512	4

\* Each injection series consisted of 5 to 7 intravenous injections of antigen. The date of bleeding was approximately 10 days after the last injection of the series.

Antibody against spore and vegetative cell antigens was present in both sera, the titer being highest with the homologous antigen. Since the rabbit immunized to the vegetative antigen had received repeated series of injections of this material, the cross reaction with the spores is not surprising. One series of injections was used with the rabbit receiving alkali-treated spores, yet the vegetative cell titer was one-fourth that of the spore titer. This is worthy of attention, first,

because, according to Krauskopf and McCoy, any free vegetative cell material in the KOH-treated spore vaccine would be eliminated by the treatment, and, second, because antiserum produced in a rabbit in response to three series of injections of untreated spores showed a vegetative cell titer only  $\frac{1}{4}$  that of the homologous spore titer. Evidently a vegetative cell antigen does exist in the spore, but in such a position or condition that rigorous treatment is required to expose it.

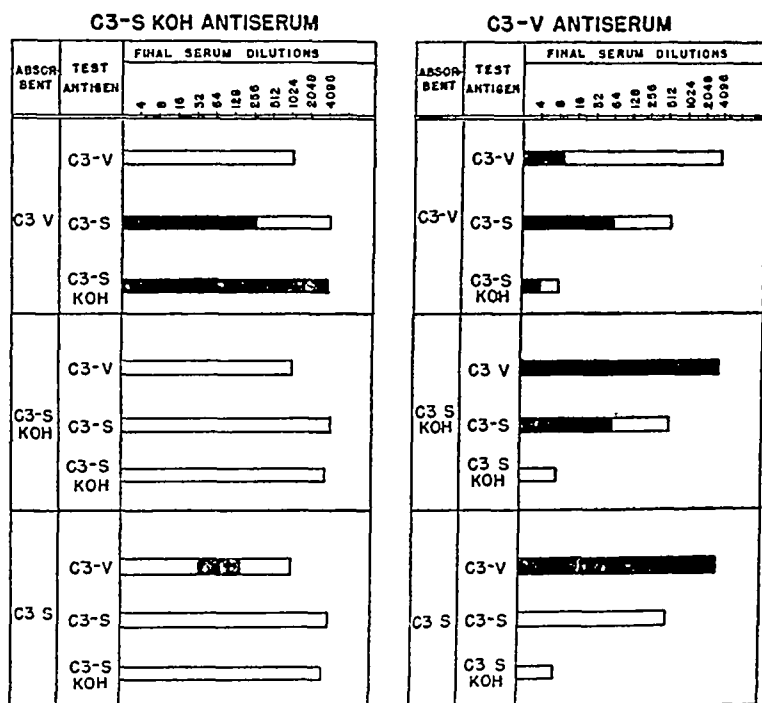


FIG 1 AGGLUTININ ABSORPTIONS OF C3-V AND C3 S KOH ANTISERA

Entire block represents titer before agglutinin absorption. Solid portions are titer after absorption.

Absorption of the antiserum against alkali-treated spores with vegetative cells partially removed antibody against treated and untreated spores. Absorption with untreated spores removed antibody to both treated and untreated spores, and to vegetative cells, though results with the vegetative cells were not consistent on repetition. Our results differed from those of Krauskopf and McCoy chiefly in the absorptive efficiency shown by the alkali-treated spores. In the present study, the KOH-treated spores were able to induce and to absorb antibody to treated and untreated spores, and to vegetative cells, whereas treated *B. niger* spores would induce antibody against all the antigens, but only absorb antibody to themselves. Thus, one or more antigenic components, demonstrable by *in vitro* methods in the case of C3, became evident only upon animal inoculation when *B. niger* was used. Either a species difference or the longer exposure

to alkali of the spores of C3 may account for this difference between our results and those of Krauskopf and McCoy. With both organisms alkali treatment has permitted detection of an otherwise obscured antigenic relationship between spores and parent vegetative cells. In view of these results and the fact that Krauskopf and McCoy did not use untreated spores to prepare a spore antiserum, their further conclusion that specific spore antigens do not exist appears to be unwarranted.

The great complexity in the antigenic pattern of the spore became even more evident when a study of interspecies agglutination was undertaken. The results are recorded in table 3. These data reveal that spores as well as bacillary forms are capable of agglutination with antisera prepared against material from other species. That this has gone unnoticed in the work of others (Howie and Cruickshank, 1940, Bekker, 1944) is probably due to the fact that species too distantly related have been used in the attempted demonstration. For example, C3 antispore serum will not clump C4 or S8 spores, but does agglutinate *B. brevis* and B40 spores to a titer approaching the homologous system.

TABLE 3  
*Heterologous spore agglutination*

ANTISERUM	SPORE ANTIGENS			
	C3 S	Ba8 (4) S	13 S	A20 S
C3-S	4,096*	2,048	0	4,096
13 S	64	128	512	4
Ba8 (4)-S	2,048	2,048	16	1,024

\* Highest serum dilution in which agglutination occurred

Extensive heterologous absorptions will be necessary to determine the number and various specificities of the spore antigens. It is already clear, however, that one of the strains, C3, contains at least three surface antigens. Two of these are involved in cross absorptions with B40 (table 4). An antigen common to the C3 and B40 spores is responsible for reciprocal cross-agglutination reactions. Absorptions show that there is a second antigen in C3, not absorbable by B40 spores. A third component is indicated by agglutinations with strain 13. In this case, C3 spores are clumped by anti-13 spore serum, whereas the spores of strain 13 are not clumped by anti-C3 spore serum. This disparity in cross agglutination can be interpreted to mean that the antigen held in common by the two strains is located on the surface of the C3 spores but is absent from the surface of strain 13 spores.

Precipitating antibody is induced by spores and can be demonstrated by the use of precipitinogens prepared by either acid (Lamanna, 1942) or formamide extraction of spores. To study the relationship of the precipitinogen to the agglutinogens of spores and vegetative cells, qualitative precipitin tests were performed on spore and vegetative cell antisera before and after absorption by spores and vegetative cells (table 5). Agglutinins and precipitins were simul-

TABLE 4  
*Antigenic relation of C3 and B40 spores*

ANTISERUM	ABSORBENT	ANTIGEN	FINAL SERUM DILUTIONS—1										C*
			4	8	16	32	64	128	256	512	1 024	2 048	
C3 S	None	C3		+	+	+	+	+	+	+	+	+	—
		B40		+	+	+	+	+	+	+	—	—	—
	B40	C3	+	+	+	+	+	+	+	+	sl +		—
		B40	—	—	—	—	—	—	—	—	—		—
B40	None	C3	+	+	+	+	+	+	+	+	—	—	—
		B40	+	+	+	+	+	+	+	+	+	—	—
	C3	C3	—	—	—	—	—	—	—	—	—	—	—
		B40	+	+	+	+	+	+	+	+	+	—	—

\* Control tube

TABLE 5  
*Tests of absorption of agglutinins and precipitins from vegetative and spore antisera by agglutinogens*

ANTISERUM	ABSORBENT	AGGLUTINATIONS		PRECIPITATIONS	
		Homologous veg etative titer	Homologous spore titer	Homologous veg etative antigen	Homologous spore antigen
SS-V no 22 7-14-47	—	4,096	64	+	+
	SS-V	0	0	0	0
	SS S	4,096	4	+	0
SS S no 7 7-14-47	—	256	256	+	+
	SS V	0	256	0	+
	SS-S	64	0	+	0
C3-V no 13 10-30-46	—	4,096	0	+	0
	C3-V	0	0	0	0
	C3 S	4,096	0	+	0
C3 S no 9 11-7-46	—	0	256	+	+
	C3-V	0	256	0	0
	C3 S	0	0	0	0
13-V 8-11-47	—	4,096	64	+	0
	13-V	0	64	0	0
	13 S	4,096	0	+	0
13 S 7-25-47	—	128	128	0	+
	13-V	0	128	0	+
	13 S	16	16	0	0

\* sl, slightly positive, (F), formamide extracts, (A), acid extracts

taneously and specifically absorbed, that is, vegetative cells absorbed vegetative cell agglutinins and precipitins, whereas spores absorbed all and only spore antibody

Absorption of agglutinins by precipitinogen was successful in two cases. Two S8 antispore sera, different bleedings from the same rabbit, were used. Absorption with formamide-extracted precipitinogen completely removed the antispore agglutinin titer (table 6). This procedure was not always so successful as for the case recorded in table 6. The difficulty increased as the agglutinin titer became higher, part, and sometimes all, of the agglutinin titer remaining even when further addition of precipitinogen caused no precipitate to appear. Thus in these cases after complete removal of precipitin even the presence of excess precipitinogen did not suppress or inhibit the agglutination reaction. The likelihood that the precipitinogen is a haptene fraction of an agglutino-gen is suggested by the complete susceptibility of the precipitating antibody to removal by absorption by agglutino-gen. That not all the agglutinogens possess the ex-

TABLE 6

*Absorption of agglutinins and precipitins from spore antisera by precipitinogens*

ANTISERUM	ABSORBENT	AGGLUTINATIONS		PRECIPITATION
		Homologous vegetative titer	Homologous spore titer	Homologous spore antigen (F)
S8-S	—	32	128	+
2- 3-47	S8 S (F)	64	4	0
S8 S	—		128	+
4-25-47	S8 S (F)		0	0

(F), formamide extract

tractable carbohydrate haptene is concluded from the cases in which the precipitin absorption was incapable of seriously reducing the agglutinin titer.

The biological significance of the data presented lies in the demonstration of the antigenic complexity of the bacterial spore. Like all other living cells the spore proves to be composed of a mosaic of antigens. Some of these antigens are characteristic of the spore, whereas others are held in common with the parent vegetative cell. The fact that the spore does possess antigens not found in vegetative cells provides definite evidence of important chemical differences in the composition of complex organic molecules of spores and vegetative cells. The demonstration of carbohydrate haptene material peculiar to spores means that the carbohydrate as well as the protein composition of spores is a proper field of study in any attempt to determine the chemical basis for the unique characteristics of the spore with regard to resistance and viability. The antigenic differences and relations between spores of various species possibly indicate a fertile and relatively ignored field of study for taxonomic purposes. From the medical point of view, the distinct antigenic qualities of bacterial spores make it theoretically necessary to consider immunity against spores apart from immunity

to vegetative cells in any serious attempts to understand infection by spore-formers, and in the development of proper specific prophylactic procedures

#### ACKNOWLEDGMENT

We wish to record our appreciation of the interested and efficient technical assistance of Mr Donald M Lowery

#### CONCLUSIONS

The bacterial spore has an antigenically complex structure, a complexity extending to the cell surface. The spore possesses antigens characteristic of itself as well as others held in common with the parent vegetative cell.

An agglutinin for spores can be demonstrated in anti-vegetative-cell serum, and by the injection of alkali-treated spores to prepare antiserum a vegetative cell agglutinin can be shown to exist in spores. The location of these common agglutinogens mostly inside the spore is suggested by two facts, first, that special procedures such as alkali treatment or extended immunization, are required to discover them, and, second, that absorption by untreated spores is specific for spore agglutinins, and absorption by living vegetative cells is specific for the vegetative cell agglutinins.

Carbohydrate type haptens that will yield precipitates in the precipitin reaction may be isolated from spores. The location of precipitinogens on the spore surface is probable, since absorption of agglutinins will also remove precipitin.

Cross reactions between spores from different species can be demonstrated by the agglutination procedure when closely related organisms are used.

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# STUDIES ON THE EFFECT OF IMMUNE REACTIONS ON THE METABOLISM OF BACTERIA

## I METHODS AND RESULTS WITH *EBERTHELLA TYPHOSA*<sup>1</sup>

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Immune defense generally results from a combination of bacterial antigen and homologous antibody with the possible intervention of lytic reactions dependent on complement and host phagocytes. The formulation of such a general concept as this, of course, reveals little about the responsible biochemical mechanisms concerned. Definition of these mechanisms challenges the present-day research worker. One such mechanism advanced by Sevag (1945) and others is the inhibition of bacterial enzymes through combination with specific antibody. Whether oxidative enzymes are included in such inhibitions has not yet been determined experimentally, but a priori one might reject their participation if it is assumed they are entirely within the cell. These experiments were made to provide information on this phase of the main problem of the production of antibody to oxidative enzymes.

### MATERIALS AND METHODS

*Typhoid cultures and the estimation of the weight of Eberthella typhosa in relation to bacterial turbidities.* Stock cultures of *Eberthella typhosa*, the nonflagellate laboratory strain O-901 and the flagellate strain H-901, were preserved in the refrigerator and transferred monthly to a fresh extract agar slant. Tryptose broth inoculated from such stocks was incubated for 8 hours at 37 C, and one loopful was used to inoculate 150 ml of extract broth, pH 7.0. After incubating for 16 hours the cells were recovered by centrifugation, washed once with 15 ml distilled water, and then uniformly suspended in distilled water. Within certain limits the dry weight was proportional to the turbidities of this suspension (in the range of 14 to 70) when measured in a Klett-Summerson colorimeter with filter 42. The linear portion of the standardization curve had a slope corresponding to one turbidity unit equivalent to 0.0141 mg bacilli in 10 ml suspension, accordingly, a turbidity of 70 of 10 ml suspension equaled 1 mg, which corresponded to  $3 \times 10^9$  bacilli by plate count.

*Measurement of oxygen consumption.* A uniform bacterial suspension was prepared in 0.033 M phosphate buffer, pH 7.2. The turbidity was determined as above by diluting 0.2 ml of the suspension to a volume of 10 ml in a standard

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<sup>2</sup> With the assistance of Evelyn R. Baker.

tube with phosphate buffer. The heavier suspension was diluted until 0.2 ml of final suspension in a volume of 10 ml gave readings corresponding to turbidities indicated in the tables as zero hour values.

*Digestion of antibody from agglutinated bacilli* Bacterial turbidities of the agglutinated systems in the presence and absence of complement were determined after the agglutinated bacteria were digested with activated papain to remove the antibody (Rosenheim, 1937, Kalmonson and Bronfenbrenner, 1943). Four grams of papain (Merck) in 100 ml of 0.066 M phosphate buffer, pH 7.2, were shaken mechanically for 90 minutes and then filtered through a filter paper and a Seitz E K filter. The filtrate, pH 6.9, was the stock solution. The papain was activated just before use by diluting the stock solution with an equal volume of 0.066 M phosphate buffer, adding 0.5 ml of 0.2 M cysteine hydrochloride per 10 ml, and incubating at 37 C for at least 60 minutes (final pH 6.65).

At the conclusion of an experiment, the grease on the inside of the mouth of the Warburg flasks was removed by a swab dipped into xylol but free from excess xylol. The contents of the flasks and the washings, using 2.5 ml of phosphate buffer, were centrifuged in an angle centrifuge for 15 minutes at 5,000 rpm. The supernatant fluids were poured off, and the sediments were washed with 4 ml of phosphate buffer and recentrifuged. The sediments were then treated with 3.5 ml of 0.033 M phosphate buffer and 1.5 ml of activated papain solution and shaken. Digestion was carried out in a 37 C water bath for 30 to 40 minutes with vigorous shaking every 10 minutes. The contents were diluted to 10 ml volume with phosphate buffer and turbidities determined. As a control, a buffer solution containing papain was treated in the same manner.

*Preparation of immune serum* White rabbits, each weighing 5 pounds, were immunized by intravenous injections of formalin-killed broth cultures of *E. typhosa*. Two-tenths ml of culture were used for the first injection. The inoculum gradually increased to 1.5 ml at the end of the sixth day. Injections were then made every other day for 5 days, using 2 ml of culture per injection. The specific immune serum vs. the flagellate strain H-901 agglutinated H-901 cells to a titer of 1:20,000 and O-901 cells to a titer of 1:5,160. The serum prepared against the aflagellate strain O-901 agglutinated O-901 cells to a titer of 1:10,000 and H-901 cells to a titer of 1:1,280. The immune sera were bottled under aseptic conditions and inactivated at 56 C for 30 minutes. No preservative was used.

Serum from the same rabbit was obtained before injections, bottled, and inactivated, and it served as the control as indicated in the tables.

*Complement* Fresh rabbit or guinea pig serum used for complement was obtained by bleeding the animal, collecting the serum, freezing overnight, and thawing before use. The complement activity of rabbit sera varied considerably, the best hemolytic titers ranging from 1:25 to 1:42, pooled guinea pig sera were fairly uniform with a titer of about 1:200. Since the source of complement apparently does not influence the respiration (see table 1), guinea pig complement was principally used.

*Calculation of  $Q_{O_2}$  values of oxygen consumption by agglutinated and lysed bacterial fractions in complement-fixing systems* Previous attempts by other in-



investigators (Sevag, 1945) to demonstrate an inhibitory effect by antibacterial immune sera on the aerobic and anaerobic respiration of bacteria have yielded results of uncertain value because of technical difficulties that arise from the

TABLE 1

*Inhibition of the oxygen consumption in the presence of glucose by E typhosa, strain O-901, by the action of antibody and complement a comparison of the activities of rabbit and guinea pig complements*

TIME	BACTERIAL TURBIDITIES*				QO <sub>2</sub> † (ml <sup>3</sup> O <sub>2</sub> /HOUR/MG BACILLI)				BACILLI LYSED‡		CHANGE IN O <sub>2</sub> UPTAKE BY LYSED BACILLI	
	Rabbit complement		Guinea pig complement		Rabbit complement		Guinea pig complement		Rabbit complement	Guinea pig complement	Rabbit complement	Guinea pig complement
	Normal rabbit serum	Anti serum	Normal rabbit serum	Anti serum	Normal rabbit serum	Anti serum (lysed bacilli)	Normal rabbit serum	Anti serum (lysed bacilli)	Rabbit complement	Guinea pig complement	Rabbit complement	Guinea pig complement
mins									%	%	%	%
0	23	23	23	23	—	—	—	—	—	—	—	—
30	23	8	23	8	246	218	282	226	65 2	65 2	12 0	20 0
90	23	5	24	5	238	115	254	116	78 2	78 2	50 4	54 3
180	23	3	25	1	211	70	211	74	87 0	94 0	66 8	64 9

Each of the respiratory systems representing columns 2, 3, 4, and 5 contained 0.2 ml of 10 per cent glucose, 0.2 ml of *E typhosa*, O-901 strain, having a turbidity represented by the zero hour turbidity readings, 1.9 ml M/30 phosphate buffer of pH 7.2. The volume of undiluted complement added, as indicated, was 0.1 ml, giving a final dilution of 1:25 in the system. As indicated, the volume of 1:10 dilution of antiserum or normal rabbit serum was 0.1 ml, giving a final dilution of 1:250 in the system. (The agglutinating titer of the immune serum was 1:10,000). A dilution of 1:250 was found to be the optimal dilution for lysis when the turbidity of the culture was 23 and the final complement dilution was 1:25.

The center cup of the Warburg flask contained a roll of filter paper wetted with 0.3 ml of 20 per cent KOH to absorb the CO<sub>2</sub> produced during the respiration.

\* One unit of bacterial turbidity measured with the Klett-Summerson photoelectric colorimeter (filter no. 42) is calculated to be equal to 0.0141 mg bacilli.

† QO<sub>2</sub> values were similar for the systems containing (a) bacilli + normal rabbit serum with active or inactive complement, and (b) bacilli + antiserum with inactive complement. The QO<sub>2</sub> values for the lysed bacilli in systems containing antiserum + active complement were calculated as described on page 3a.

‡ The turbidity reading of the inoculum at 0 hour was compared with the readings given in columns 3 and 5.

absence of a practical method for the estimation of the weight of bacteria present in agglutinated bacterial clumps.<sup>3</sup> The manner in which the methods described

<sup>3</sup> Oldfelt (1942) has recently attempted without success to determine the relative amounts of bacterial and antibody nitrogens present in agglutinated bacterial clumps, in a manner comparable to the techniques used by Heidelberger and Kabat (1934, 1936) for the determination of the amount of antibody combined with agglutinated bacteria. Oldfelt resorted, therefore, to the use of high dilutions of immune serum, and an excess of complement. Under these conditions he stated that he found no increase in the nitrogen content

above are applied to the actual data is illustrated by the following detailed calculation

*Calculation of  $\text{mm}^3 \text{O}_2/\text{hour}/\text{mg}$  ( $Q_{\text{O}_2}$ ) of lysed and unlysed bacterial fractions in complement fixing systems* The turbidity of bacteria in lytic and nonlytic systems was measured after papain digestion with the Klett-Summerson photoelectric colorimeter using no. 42 filter. A turbidity of 70 corresponds to 1 mg bacilli, or a unit turbidity represents 0.0141 mg bacilli. The following is a detailed example of the calculations used in this study

A Bacterial turbidity in the reaction systems at zero hour was 23

Fifteen minutes after zero hour the following results were obtained

B Bacilli + normal rabbit serum + active complement Turbidity = 26,  $\text{mm}^3 \text{O}_2$  consumed = 35,  $\text{mm}^3 \text{O}_2/\text{unit turbidity} = 1.34$

C Bacilli + immune rabbit serum + inactive complement Turbidity = 25,  $\text{mm}^3 \text{O}_2$  consumed = 30,  $\text{mm}^3 \text{O}_2/\text{unit turbidity} = 1.20$

D Bacilli + immune rabbit serum + active complement Turbidity = 15,  $\text{mm}^3 \text{O}_2$  consumed = 31,  $\text{mm}^3 \text{O}_2/\text{unit turbidity}$ , see below

*Calculation of  $Q_{\text{O}_2}$  of the reaction system B*

Mg bacilli/unit turbidity  $\times 26 =$  mg bacilli in system B, or  $0.0141 \times 26 = 0.3666$  mg bacilli

$\text{mm}^3 \text{O}_2$  consumed by system B/0.3666, or  $35/0.3666 = 95 \text{ mm}^3 \text{O}_2$  consumed by 1 mg bacilli in 15 minutes

Therefore,  $Q_{\text{O}_2} = 95 \times 60/15 = 380 \text{ mm}^3 \text{O}_2/\text{hr}/\text{mg bacilli}$

*Calculation of  $Q_{\text{O}_2}$  of the reaction system C*

As in B,  $0.0141 \times 25 = 0.3525$  mg bacilli present in system C

$\text{mm}^3 \text{O}_2$  consumed by 0.3525 mg bacilli was 30, or  $30/0.3525 = 85 \text{ mm}^3 \text{O}_2/\text{mg bacilli}/15$  minutes

$Q_{\text{O}_2} = 85 \times 60/15 = 340 \text{ mm}^3 \text{O}_2/\text{hr}/\text{mg bacilli}$

*Calculation of  $Q_{\text{O}_2}$  of lysed bacilli of the reaction system D*

Since the  $Q_{\text{O}_2}$  values in system B containing bacilli and normal rabbit serum + active complement and in system C containing bacilli + immune rabbit serum + inactive complement are similar, the assumption is made that the unlysed bacilli in system D use the same volume of  $\text{O}_2$ , per unit turbidity, as the bacilli in systems B and C. Therefore the calculation of  $Q_{\text{O}_2}$  value for the lysed bacilli in system D is based on a comparison with systems B and C

Lysed fraction of bacilli corresponds to the difference between the bacterial turbidity 23 at zero hour and the turbidity 15 measured, for example, after a 15 minute reaction period.  $23 - 15 = 8$  units of turbidity representing the lysed fraction of bacilli or 0.1123 mg lysed cells

Total  $\text{mm}^3 \text{O}_2$  consumed by system D = 31

$31 \text{ mm}^3 \text{O}_2 - 20.1 \text{ mm}^3 \text{O}_2 (= 1.34 \text{ mm}^3 \text{O}_2/\text{unit turb} \text{ (as in system B)} \times 15, \text{ turbidity of system D}) = 10.9 \text{ mm}^3 \text{O}_2$ , or  $10.9/0.1123 \text{ mg lysed bacilli} = 97 \text{ mm}^3 \text{O}_2/15 \text{ minutes}$   
or  $31 \text{ mm}^3 \text{O}_2 - 18.0 \text{ mm}^3 \text{O}_2 (= 1.20 \text{ mm}^3 \text{O}_2/\text{unit turb} \text{ (as in system C)} \times 15, \text{ turbidity of system D}) = 13.0 \text{ mm}^3 \text{O}_2$ , or  $13.0/0.1123 \text{ mg lysed bacilli} = 115 \text{ mm}^3 \text{O}_2/15 \text{ minutes}$

$Q_{\text{O}_2} = 97 \times 60/15 = 388$

$115 \times 60/15 = 460$

of washed bacterial sediment obtained by centrifuging the contents of Warburg flasks at various periods. He expressed the final values in terms of  $\text{mm}^3 \text{O}_2/\mu\text{g}$  bacterial nitrogen (Oldfeldt, pp 57-70). Since he did not take into account the volume of oxygen consumed by the lysed fractions of bacilli, his method of determining values would be inaccurate. As will be seen from the results we report here, the lysed fractions of bacilli consume oxygen in the presence and absence of a substrate, very often in amounts greater than those used by unlysed bacilli, followed by a gradual decrease in oxygen uptake.

## EXPOSITION AND DISCUSSION OF RESULTS

*Measurement of oxygen consumption by E typhosa in systems containing normal rabbit and homologous immune rabbit serum* The results presented as representative data in table 2 show that in no instance, in the presence or absence of glu-

TABLE 2

*Inhibition of oxygen consumption in the presence of glucose by E typhosa, strain O-901, by the action of antibody and guinea pig complement*

A  Time	BACTERIAL TURBIDITIES*			QO <sub>2</sub> † (MM <sup>3</sup> O <sub>2</sub> /HOUR/MG BACILLI)			BACILLI LYSED‡	CHANGE IN O <sub>2</sub> UPTAKE BY LYSED BACILLI
	Bacilli normal rabbit serum + active com- plement	Bacilli immune rabbit serum + inactive com- plement	Bacilli immune rabbit serum + active com- plement	Bacilli normal rabbit serum + active com- plement	Bacilli immune rabbit serum + inactive complement	Immune rabbit serum + active com- plement		
	(1)	(2)	(3)	(4)	(5)	Lysed bacilli		
min							%	%
0	23	23	23	—	—	—	—	—
15	26	26	15	380	360	528	34	-39
30	27	26	8	342	332	366	65	-7
45	27	26	4	314	307	262	82	16
60	27	27	5	299	283	192	80	36
90	27	27	1	280	269	140	96	50
180	29	27	1	235	270	69	96	70

## B Systems in which rabbit complement was used

0	24	24	24	—	—	—	—	—
90	26	26	4	240	192	49	83	79
180	28	28	4	152	142	24	83	84
300	27	25	4	138	148	19	83	86

Experimental conditions identical to those described in the footnote to table 1 were used

\* One unit of bacterial turbidity measured with the Klett-Summerson photoelectric colorimeter is calculated to be equal to 0.0141 mg bacilli

† QO<sub>2</sub> values of the lysed fractions of bacilli in the systems containing immune serum and active complement were obtained by comparing these figures with those of the systems containing normal rabbit serum and active complement. This system was used since control experiments with the immune serum and inactive complement (an aliquot of active complement heated in a 56 C water bath for 30 minutes) gave results quite similar to those obtained in the normal rabbit serum and active complement system

‡ The turbidity reading of the inoculum at 0 hour was compared with the readings given in column 3. Minus sign before figures indicates increased oxygen uptake, figures without sign indicate inhibition of oxygen uptake

cose, do the QO<sub>2</sub> values for the agglutinated *E typhosa*, flagellate strain O-901, differ significantly from those of the controls containing normal rabbit serum

Homologous immune serum did not produce a significantly inhibitory effect on the oxygen consumption by H-901 cells. The sensitized cells likewise did not experience either a significant decrease in oxygen uptake or lysis when incubated with active complement. H-901 bacilli treated with specific serum and,

after refrigeration overnight, treated with complement, likewise did not experience either lysis or decrease in oxygen uptake. However, H-901 bacilli, treated with homologous immune serum and complement and refrigerated overnight, at the end of a 3-hour respiration period were found to have undergone 56 per cent lysis and showed marked decrease in the volume of oxygen consumed. For the unlysed and lysed fractions of bacilli  $Q_{O_2}$  values were, respectively, 212 and 86. That is, in comparison with the unlysed fraction, or the control (containing normal rabbit serum + active complement + H-901 bacilli), the lysed fraction of bacilli had experienced 58 per cent inhibition of the respiratory activity.

In another set of experiments the effect of anti-H-901 immune serum on O-901 bacilli, and conversely the effect of anti-O-901 immune serum on H-901 bacilli, was studied. The system containing O-901 bacilli + undiluted H 901 antiserum + inactive complement after a 3-hour period showed a turbidity of 31 and had a  $Q_{O_2}$  value of 247. The same system containing active complement (in place of inactive complement) showed a turbidity of 10 (54 per cent lysis) with a  $Q_{O_2}$  value of 126 for the lysed fraction and 246 for the unlysed fraction of O-901 bacilli, or 50 per cent inhibition of the oxygen uptake of the lysed O 901 cells by the action of H-901 antiserum and complement.

On the other hand, the H-901 bacilli (zero hour turbidity, 23) treated with O-901 antiserum and active complement, refrigerated overnight, and tested for respiratory activity, after a 3-hour period showed a turbidity of 32 and the  $Q_{O_2}$  was 165, with inactive complement the turbidity was 31 and  $Q_{O_2}$  201. Thus, despite marked agglutination, little or no lysis took place and there was only a difference of 18 per cent in the oxygen uptake. When H-901 bacilli were refrigerated with O-901 antiserum overnight and when complement was added just before the start of respiration measurements, with the inactive complement the turbidity was 37 and  $Q_{O_2}$  187, with the active complement the turbidity was 31 and  $Q_{O_2}$  193. The latter results were also obtained when the before-mentioned reagents were mixed and the respiration measurements made at once.

The flagellate strain H-901 is clearly less susceptible to lysis by the sensitizer-complement system than is the nonflagellate variant strain O-901. Alterations in oxygen consumption under the various experimental conditions cited occurred only when lysis was brought about by the action of sensitizer and complement. When lysis did not occur, specific combination with immune serum was without demonstrable effect on oxygen consumption.

Rough and smooth strains of pneumococcus type I cultures were mixed with homologous antisera (prepared against these organisms in rabbits) in the presence and absence of complement. There was no significant difference in oxygen consumption in any instance. No lysis occurred, of course. Controls containing normal rabbit serum in place of the antiserum gave similar results.

These results show that the substrates used are not only capable of diffusing through the agglutinated clumps of bacteria but also of permeating the layer of antibody molecules and associated serum components deposited on the antigenic components of the cell wall (Heidelberger and Kabat, 1934, 1936, Mudd and Anderson, 1944) and capsule (Mudd, Heinmets, and Anderson, 1943). It would

appear that there are spots on the cell wall not occupied by antibody molecules that readily permit the penetration of the substrates to the site of the active groups of the respiratory enzymes

*An analysis of the inhibition of oxygen consumption in the complement-fixing systems* A survey of the tables from 1 to 5 shows that in all instances there is a marked reduction in the uptake of oxygen by the lysed fraction of sensitized bacilli. It is to be noted, however, that in the presence of glucose, despite from 65 to 75 per cent lysis of bacilli in the 30- and 60-minute periods, there is either an insignificant degree of inhibition or a marked acceleration in oxygen uptake. Following these initial periods, the degree of lysis remaining practically unchanged, the oxygen consumption shows a marked reduction (table 2, table 3, exp 93, table 4, exp 112, table 5, exp 109 and 111).

One may postulate that there are opposing reactions concurrently consuming oxygen. During the initial periods, the reactions that are inhibited would appear to proceed at a slower rate, a fact that may account for the absence of reduction of total oxygen consumption during these initial periods. The reason that reduction of oxygen consumption becomes marked as the reactions progress may be due to the oxidation of glucose breakdown products, which may appear to be principally susceptible to the inhibitory action of the system. In this connection the results of the following two types of experiment may be of some interest.

*Increase of inhibition of respiration during the early periods of respiration associated with increased oxygen uptake in the presence of yeast extract* The results of several experiments with yeast extract are presented in table 3. It can be seen that in the presence of yeast extract the  $Q_{O_2}$  values of the controls are from 17 to 30 per cent higher than in its absence (table 3, exp 93 (2)). In the presence of yeast extract, antibody and complement, the reductions in oxygen uptake are from 27 to 40 per cent greater than in its absence. At the end of the 60-minute period, in the system without yeast extract the oxygen uptake of lysed bacilli shows a 7 per cent greater value and, in contrast, a 20 per cent inhibition in its presence. In another experiment (table 3, exp 91) at the end of 90- and 180-minute periods, the inhibitions were, respectively, 17 and 35 per cent, and in the presence of yeast extract 40 and 72 per cent, showing from 106 to 136 per cent greater inhibition. One important effect of the factors in yeast extract may, therefore, be to hasten or to involve those reactions that are susceptible to the inhibitory action of the system (table 3, exp 93 (2), 91, and 95). Since the acceleration of the oxidation of glucose results in the speedier formation of breakdown products, such as glycerol, in parallel tests the rate and degree of oxygen uptake of the systems containing glucose and glycerol were determined. The results are discussed below.

*Increase of inhibition during the earlier periods associated with increased oxygen uptake in the presence of glycerol* As considered above, the initiation of greater inhibitory effect following lysis on oxygen uptake at an earlier period in the presence of yeast extract may be considered to be related to the correspondingly faster formation of one or more critical breakdown products. Accordingly, the inhibition of the oxidation of glycerol, for example, should start sooner and show a

TABLE 3

Comparison of the oxygen consumption by *E. typhosa* and degree of inhibition in the presence and absence of yeast extract

TIME	NORMAL RABBIT SERUM + COMPLEMENT				IMMUNE SERUM + COMPLEMENT				BACILLI LYSED		CHANGE IN O <sub>2</sub> UPTAKE BY LYSED BACILLI	
	Bacterial turbidity		QO <sub>2</sub>		Bacterial turbidity		QO <sub>2</sub> lysed bacilli					
	Yeast Extract		Yeast Extract		Yeast Extract		Yeast Extract		Yeast Extract		Yeast Extract	
	None	0.2%	None	0.2%	None	0.2%	None	0.2%	None	0.2%	None	0.1%

Exp 93(2)

min									%	%	%	%
0	24	24			24	24						
60	26	40	226	294	9	6	245	236	62.5	75	-7	20
120	28	55	196	240	9	6	118	106	62.5	75	40	50
180	29	60	177	207	5	6	88	64	80.0	75	51	70

Exp 93(1)

	NORMAL RABBIT SERUM + BUFFER				IMMUNE SERUM + BUFFER						CHANGE IN O <sub>2</sub> UPTAKE BY INTACT BACILLI	
0	23	23			23	23						
180	29	54	135	185	30	56			0	0	0	0

Exp 91

	NORMAL RABBIT SERUM + COMPLEMENT				IMMUNE SERUM + COMPLEMENT						CHANGE IN O <sub>2</sub> UPTAKE BY LYSED BACILLI	
0	24	24			24	24						
90	25	47	209	257	7	6	174	154	71	75	17	40
180	27	51	182	236	4	5	118	66	83	80	35	72

Exp 95 \*

	NORMAL RABBIT SERUM + COMPLEMENT				IMMUNE SERUM + COMPLEMENT							
0		23				23						
15		24		460		19		?		17		?
30		26		370		14		220		40		40.5
45		25		394		7		207		70		47.4
60		30		340		5		181		78.3		47.0
90		40		280		2		162		91.3		42.0
180		57		211		1		84		95.6		60.0

For flask contents see footnotes to tables 1 and 5

\* Turbidities obtained with papain digestion (exp 91 and 93), as will be seen, are comparable with those obtained without papain digestion (exp 95)

greater degree of inhibition. A survey of the results presented in table 4 (exp 112 and 113) shows that in the presence of glucose at the end of a 1-hour period there is no inhibition, in contrast, a 14 per cent increase in oxygen uptake is to be noted. In the presence of glycerol during the same period 56 per cent in-

TABLE 4

*A comparison of the oxygen consumption by E typhosa in complement-fixing lytic systems in the presence of glucose or glycerol and in the absence of an added substrate*

TIME	NORMAL RABBIT SERUM + COMPLEMENT		IMMUNE SERUM + COMPLEMENT		LYSED BACILLI	CHANGE IN O <sub>2</sub> UPTAKE BY LYSED BACILLI
	Bacterial turbidity	QO <sub>2</sub>	Bacterial turbidity	QO <sub>2</sub> lysed bacilli		
Exp 112 Systems containing glucose						
min					%	%
0	23		23			
15	25	156	25		0	
30	26	222	26		0	
60	29	220	8	250	65	-14
Exp 112 Systems containing glycerol						
0	23		23			
15	25	260	10	348	60	-34
30	25	322	9	322	61	0
60	26	321	4	141	82	56
Exp 113 Systems containing glycerol						
0	24		24			
60	27	384	6	113	75	70
120	31	311	4	55	83	82
Exp 113 Systems without added substrate						
0	24		24			
60	27	204	15	141	37.5	31
120	33	153	11	82	54.0	46
Exp 114 Systems without added substrate						
0	24		24			
60	29	190	20	195	17.0	-3
120	31	162	9	90	62.5	44

For the contents of the flasks see footnotes to tables 1 and 5

hibition occurred. Similarly, a comparative survey of the results presented in table 5 (exp 109) shows that at the end of the 1-hour period there is only 14 per cent inhibition in the presence of glucose. In the presence of glycerol the inhibition is 70 per cent, which means that the system is severalfold more effective

against glycerol than against glucose oxidation. In another experiment (table 5, exp 111) at the end of 90 minutes the oxidation of glucose was inhibited only 3 per cent and that of glycerol 77 per cent. The significance of these comparative results obtained in parallel tests is emphasized by the fact that the oxidation of glycerol is a much more active process than that of glucose. As will be seen (table 4, exp 112), the  $Q_{O_2}$  value for glucose is 220, and for glycerol 321 at the

TABLE 5

*A comparison of the oxygen consumption by E typhosa in complement fixing systems in the presence of glucose and glycerol*

TIME	NORMAL RABBIT SERUM + COMPLEMENT		IMMUNE SERUM + COMPLEMENT		LYSED BACILLI	CHANGE IN $O_2$ UPTAKE BY LYSED BACILLI
	Bacterial turbidity	$Q_{O_2}$	Bacterial turbidity	$Q_{O_2}$ lysed bacilli		
Exp 109 Systems containing glucose						
<i>min</i>					%	%
0	24		24			
60	25	252	7	216	70.8	14
120	28	213	5	113	80.0	17
Exp 111 Systems containing glucose						
0	23		23			
90	26	218	5	212	78	3
180	32	167	2	117	91	30
Exp 109 Systems containing glycerol						
0	24		24			
60	25	360	6	110	75	70
120	25	334	5	39	80	88
Exp 111 Systems containing glycerol						
0	23		23			
90	23	359	3	82	90	77
180	26	276	1	56	96	80

Reaction systems consisted of 0.2 ml of 10 per cent sterile filtered glucose or glycerol, 0.1 ml of 1 to 10 dilution of antiserum, 0.1 ml of undiluted active guinea pig complement, 1.9 ml of M/30 phosphate buffer of pH 7.22, and 0.2 ml of bacterial suspension (in side arm). The center cup contained a roll of filter paper (no. 40) soaked with 0.3 ml of 20 per cent KOH.

end of a 1-hour period. Similarly, at the end of the 90-minute period (table 5, exp 111) the  $Q_{O_2}$  value for glucose is 218, and for glycerol 359. That is, glycerol uses 50 to 65 per cent more oxygen than glucose. (For a recent study on the oxidation of glycerol see Mickelson and Shideman, 1947.) In conclusion, the systems that consumed a greater volume of oxygen, as in the presence of cell extract, showed also a greater degree of susceptibility to the action of the inhibitory factors of the systems following lysis.



*Relation of the rate of oxygen consumption to the rate of lysis* The results presented here show that *E. typhosa*, strain O-901, lyses in a complement-fixing system in the absence of an added substrate, on the average from 28 to 58 per cent within 60 to 120 minutes (table 4, exp 113 and 114). In the presence of glucose or glycerol as a substrate, the lysis of bacilli during the same period is from 70 to 80 per cent (table 5, exp 109 and 111). In parallel tests, the rate of lysis in the presence of glucose and glycerol was determined. It will be seen (table 4, exp 112) that at the end of the first 30-minute period there was no lysis in the presence of glucose, and the lysis at the end of the first 60-minute period was 65 per cent. During the same periods, the lysis in the presence of glycerol was from 60 to 82 per cent. In fact, in contrast to the absence of lysis in the presence of glucose at the end of the first 15 minutes there was 60 per cent lysis when the system contained glycerol. These results may be interpreted to indicate that the rate of lysis in the three different systems mentioned above is in the following order

glycerol > glucose > absence of added substrate

In control systems, the degree of growth in each system is practically identical. The previously mentioned differences in the rate of lysis of bacilli do not therefore appear to be related to growth activities (table 4, column 2). On the other hand, a comparison of  $Q_{O_2}$  values for the 1-hour period shows values of from 321 to 384 for glycerol, 220 for glucose, and from 190 to 204 for the system not containing added substrate. Also the order of oxygen-consuming activities is comparable with the degree of lysis in the presence of the substrates named above. This may indicate that certain oxidation-reduction reactions play a role in regulating the rate of lysis of bacilli (Ecker, Pillemer, and Werthheimer, 1938). In the light of the foregoing considerations it would appear that the reactions participating in the dehydrogenation of glycerol maintain the lytic system at a reduced state to a greater degree than do the other two systems, which may account for the accelerated rate of lysis in the presence of glycerol.

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#### SUMMARY AND CONCLUSIONS

The effect of homologous immune serum with and without complement on the oxygen consumption by *Eberthella typhosa* (strains O-901 and H-901) and pneumococcus has been studied.

A method has been worked out that makes it possible to calculate  $Q_{O_2}$  values ( $\text{mm}^3 \text{O}_2$  per mg bacteria per hour) for intact, agglutinated, and lysed fractions of bacteria. The values thus obtained make it possible to evaluate the effect of various agents on the oxygen consumption of bacteria under the influence of immune and other factors.

Agglutinated *E. typhosa* (and pneumococci) consume volumes of oxygen equal

to those of the respective controls. Intact sensitized cells with or without complement do not experience loss of oxidative activity, indicating that the formation of agglutinated clumps of bacteria does not involve physical or immunological barriers to the activity of oxidative enzymes.

Sensitized *E. typhosa* (O-901) cells acted upon by complement undergo lysis. Immediately after lysis considerably more oxygen is used than by the controls containing the intact cells. Subsequently, the oxygen consumption of the lysed system undergoes up to 88 per cent reduction.

In simultaneous tests, oxygen consumption in the presence of yeast extract and glucose is greater than in the presence of glucose alone. In the presence of yeast extract, the reduction in oxygen uptake following lysis is likewise greater. In the presence of glycerol the oxygen consumption is also markedly greater than in glucose alone. The reduction in oxygen uptake by the glycerol containing system is likewise much greater and more prompt than in glucose alone.

Whether or not the previously mentioned reduction in oxygen uptake by the lysed fractions of bacilli obtained by the action of complement on sensitized cells is due to the deterioration of liberated enzyme systems or to an inhibitory effect of a specific combination between liberated intact oxidative respiratory enzymes and homologous antibodies cannot at present be answered. Further work pertaining to this question is in progress.

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# SEROLOGICAL AND BIOLOGICAL CHARACTERISTICS AND PENICILLIN RESISTANCE OF NONHEMOLYTIC STREPTOCOCCI ISOLATED FROM SUBACUTE BACTERIAL ENDOCARDITIS

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The finding of a new variety of nonhemolytic streptococcus, designated as *Streptococcus s b e*, has been recently reported (Loewe, Plummer, Niven, and Sherman, 1946). The organism was found 41 times in 113 cultures from 100 cases of subacute bacterial endocarditis. Certain significant cultural and biochemical characteristics were attributed to this new variety as well as a refractoriness to penicillin therapy. However, no data on the *in vitro* sensitivity to penicillin were included in the report. The organism was subsequently studied in great detail (Niven and White, 1946, Washburn, White, and Niven, 1946, White and Niven, 1946, Niven, Kizuta, and White, 1946), and a considerable degree of homogeneity was attributed to it. Of particular significance was the report (Washburn, White, and Niven, 1946) that all 42 strains of *Streptococcus s b e* studied fell into two distinct serological types as determined by the precipitin method. Five of these strains gave positive tests with both type I and type II antisera. An antiserum prepared by immunizing rabbits with one of the five strains reacted with extracts of all the strains of *Streptococcus s b e* of types I and II. This was believed to be due to the presence of specific components of both types in the cells of the polyvalent strain, rather than to the presence of a common antigenic component.

The grouping of nonhemolytic streptococci on a serological basis, similar perhaps to the Lancefield grouping of the hemolytic streptococci, would be a logical goal since classification based upon cultural and biochemical characteristics has many deficiencies, for example, lack of specificity and the inconstancy of some of the reactions. Soloway (1942) attempted to group serologically the streptococci isolated from cases of subacute bacterial endocarditis and from the human throat and extracted teeth. She found that about two-thirds of all the strains could be differentiated into 14 serological types of which about 50 per cent of the reacting strains from each source fell into two types. No serological differentiation could be shown between the streptococci from subacute bacterial endocarditis and those from the throat and teeth. No correlation of the serological with the biochemical characteristics of the various strains was found.

Dawson, Hobby, and Lipman (1944) investigated the sensitivity to penicillin of 50 strains of nonhemolytic streptococci isolated from subacute bacterial endocarditis cases. The organisms were described grossly as "viridans," "indifferent," and "resembling enterococci." Their sensitivities were expressed in relation to a standard hemolytic streptococcus strain. No findings characteristic

for any particular group could be demonstrated. They concluded that with occasional exceptions, strains of nonhemolytic streptococci isolated from cases of subacute bacterial endocarditis are sensitive to penicillin *in vitro* and that the degree of sensitivity of any particular strain does not correspond with its serological or cultural properties.

The present study on all strains of nonhemolytic streptococci isolated from subacute bacterial endocarditis in this hospital for a period of the last 20 months, concerns itself with the correlation of the serological and biological characteristics of the bacteria with their resistance to penicillin *in vitro*. The results are embodied in the following report.

#### CRITERIA USED FOR CLASSIFICATION OF ORGANISMS ISOLATED

Soon after original isolation, all organisms were subjected to serological and biological tests and their resistance to penicillin was determined *in vitro*. Thirty-four strains isolated from 32 cases were the subject of this investigation. Four strains were isolated from two recurrent infections (strains 17 and 18, and strains 19 and 20). It may be noted from table 1 that in both patients with recurrences, neither was infected with a *Streptococcus s b e* strain.

It has long been recognized that the classification of the nonhemolytic streptococci is far from satisfactory. Prior to the identification of the *Streptococcus s b e* group, only a few other groups had been more or less clearly differentiated, such as the *Streptococcus salivarius*, the enterococcus groups, and a few others. In order to facilitate the analysis of the significant characteristics of the various strains isolated, the organisms were classified by the following properties:

*Streptococcus s b e* group. Precipitation with *Streptococcus s b e* antiserum, fermentation of inulin with acid production, hydrolysis of arginine, growth on 40 per cent bile blood agar, absence of mucoid colonies on 5 per cent sucrose agar, and the synthesis of slime in 5 per cent sucrose broth.

*Streptococcus salivarius* group. Formation of large mucoid colonies on 5 per cent sucrose agar, no change in color on blood agar, failure to hydrolyze arginine, and the ability to ferment inulin and raffinose with the production of acid (Sherman, Niven, and Smiley, 1943).

*Enterococcus* group. Precipitation with Lancefield group D streptococcus antiserum, marked resistance to penicillin, growth at 45 C, and growth in media containing 6.5 per cent NaCl, and in 40 per cent bile (Evans and Chinn, 1947).

*Unclassified Streptococcus* group. The organisms that did not possess the specific characteristics described above.

Accordingly, in this study, of the 34 strains isolated, 9 were *Streptococcus s b e*, 2 each were *Streptococcus salivarius* and enterococci, and the remaining 21 were considered to be in the heterogeneous group, unclassified streptococci.

#### SEROLOGICAL CHARACTERISTICS

All strains were studied serologically. The methods employed for the preparation of the precipitating antisera and the carbohydrate extract from the organisms as well as the technique for the precipitin test, are those described

TABLE 1

Characteristics of nonhemolytic streptococci isolated from subacute bacterial endocarditis

STRAIN	PRECIPITATION WITH ANTISERUM			BIOLOGICAL CHARACTERISTICS									PENICILLIN RESISTANCE (X standard)
	Str s b e	Str 15	Str Lan D	Greening—24 hr	Fermentation— inulin	Fermentation— raffinose	Growth—45 C	Growth—65 % NaCl	Arginine hydrolysis	Growth—40% bile blood agar	5% Sucrose agar— mucoid colonies	5% Sucrose broth— slime synthesis	
<i>Streptococcus s b e</i>													
1	+	-	-	+	+	-	+	-	+	+	-	+	2
2	+	±	±	+	-	-	+	-	+	+	-	+	5
3	+	-	-	+	+	-	+	-	+	-	-	+	2
4	+	-	-	+	-	+	+	-	+	+	-	+	10
5	+	-	-	+	+	-	+	-	+	+	-	+	10
6	+	-	-	+	+	-	-	-	+	+	-	±	10
7	+	-	-	+	-	-	-	-	+	+	-	±	2
8	+	-	-	+	+	-	+	-	+	+	-	+	15
9	+	-	±	+	-	-	+	-	-	+	-	+	10
<i>Streptococcus salivarius</i>													
10	-	-	-	±	+	+	+	-	-	+	+	+	5
11	-	±	+	-	+	-	+	-	-	+	+	+	15
<i>Enterococcus</i>													
12	-	-	+	+	-	-	+	+	+	+	+	+	150
13	-	-	+	+	-	-	+	+	+	+	+	-	250
<i>Unclassified streptococci</i>													
14	-	-	-	+	-	-	-	-	+	-	+	+	2
15	-	+	-	+	-	+	+	-	-	-	-	-	5
16	-	+	±	-	-	-	+	-	-	-	+	-	5
17	-	+	±	-	-	-	+	-	-	-	-	-	1
18	-	±	±	+	-	-	+	-	-	-	-	-	5
19	-	-	-	-	+	-	±	-	+	+	-	-	2
20	-	-	-	-	-	-	+	-	-	±	-	-	1 5
21	-	-	-	+	-	+	+	-	+	-	-	-	1
22	-	-	-	-	-	-	-	-	-	-	-	-	10
23	-	-	-	+	-	-	-	-	-	-	-	-	2 5
24	-	±	-	-	+	+	-	-	+	-	-	-	5
25	-	-	-	-	-	+	+	-	+	-	-	±	5
26	-	-	-	+	-	-	-	-	+	-	-	+	5
27	-	-	-	+	-	-	-	-	+	-	-	-	5
28	-	-	-	+	-	-	+	-	+	-	-	-	10
29	-	±	±	+	-	-	+	-	-	+	-	-	2
30	-	-	-	+	-	-	+	-	-	-	-	+	10
31	-	-	-	-	-	-	-	-	-	+	-	-	15
32	-	-	±	+	+	-	-	-	+	+	-	+	5
33	-	+	-	+	-	-	+	-	+	-	-	-	1 5
34	-	±	±	+	-	-	+	-	-	-	+	+	5

+ = Strongly or definitely positive, ± = weakly or doubtfully positive, - = negative

by Lancefield (1933) Antisera were prepared for three strains (1) *Streptococcus sanguis* (*Streptococcus s b e*), a strain obtained from Washburn that contains both types I and II antigen, (2) a nonhemolytic streptococcus strain in the unclassified group (strain 15), which was isolated from an early case and which did not possess any of the significant biological characteristics ascribed to *Streptococcus sanguis*, and (3) a Lancefield group D hemolytic streptococcus (strain C1) Heat-killed organisms, as recommended by Washburn, were used to prepare the first two antisera, whereas formalin-killed organisms were employed to produce the Lancefield group D antiserum The precipitin test was conducted with varying amounts of antigen and a constant amount of serum All tests were accompanied by controls using normal rabbit serum and extract, and the various antisera without any extract, for comparison

As may be seen from tables 1 and 2, the *Streptococcus s b e* is a distinct serological entity All *Streptococcus s b e* strains reacted strongly only with the antiserum specific for the group Extracts prepared from streptococci in the

TABLE 2

*Precipitin reactions of extracts of nonhemolytic streptococci from subacute bacterial endocarditis*

EXTRACTS TESTED	ANTISERUM					
	<i>Streptococcus s b e</i>		<i>Streptococcus 15</i>		Lancefield D <i>Streptococcus</i>	
	pos	neg	pos	neg	pos	neg
<i>Streptococcus s b e</i>	9	0	1±	8	2±	7
<i>Streptococcus salivarius</i>	0	2	1±	1	1	1
<i>Enterococcus</i>	0	2	0	2	2	0
Unclassified streptococci	0	21	1± 4±	13	6±	15

other groups failed to precipitate with *Streptococcus s b e* antiserum These results confirm the observations of Washburn *et al* on the serological homogeneity of the *Streptococcus s b e* cultures

No single biological characteristic specific for any one variety could be demonstrated, with one exception. The organisms of the enterococcus group were the only ones found able to grow in 6.5 per cent NaCl broth. However, the presence of a combination of concomitant characteristics was highly suggestive for the *Streptococcus s b e* group, namely, growth on 40 per cent bile blood agar, hydrolysis of arginine, fermentation of inulin with the production of acid, absence of mucoid colonies on 5 per cent sucrose agar, and the synthesis of slime in 5 per cent sucrose broth. One unclassified streptococcus, strain 32, possessed all these properties although an extract prepared from it failed to precipitate with specific *Streptococcus s b e* antiserum.

Good correlation between the biological and serological characteristics were

TABLE 3

*Biological characteristics of nonhemolytic streptococci from subacute bacterial endocarditis*

CHARACTERISTICS	WHITE & NIVEN	STREPTOCOCCUS S.B.E (9)	STREPTOCOCCUS SALIVARIUS (2)	ENTEROCOCCUS (2)	UNCLASSIFIED STREPTOCOCCI (21)
	<i>per cent</i>	<i>no</i>	<i>no</i>	<i>no</i>	<i>no</i>
Greening—24 hr	98.0	9	1±	2	13
Fermentation inulin	81.0	5	2	0	3
Fermentation raffinose	33.0	1	1	0	4
Growth—45 C	45.0	7	2	2	12
					1±
Growth—6.5% NaCl	0.0	0	0	2	0
Arginine hydrolysis	100.0	8	0	2	9
Growth—40% bile blood agar	72.0	8	2	2	4
					1±
5% Sucrose agar—mucoid colonies	0.0	0	2	2	3
5% Sucrose broth—slime synthesis	95.0	7	2	1	5
		2±			1±

demonstrable for two groups, *Streptococcus s b e* and the enterococci. Table 3 summarizes the significant biological characteristics of the strains of each group isolated in this series.

#### PENICILLIN RESISTANCE

Resistance to penicillin *in vitro* was determined for all streptococci isolated. A tube dilution method using fresh meat extract broth as the medium and the standard *Staphylococcus aureus* H strain for comparison was employed. The tests were run in 2-ml amounts. Two series of penicillin dilutions varying from 0.5 to 0.005 Oxford units per ml were set up. Tubes were inoculated with suitably diluted 6-hour cultures of the streptococcus and the staphylococcus to give final concentrations of 600 to 800 and 200 to 300 organisms per ml, respectively, at zero hours. If no inhibition proved to be present in this range, a greater amount of penicillin was used for the streptococcus series. The tubes were incubated at 37 C overnight and the end points of complete inhibition were noted the following morning. The amount of penicillin required to inhibit

the streptococci divided by the amount necessary to inhibit the standard organism was the penicillin resistance expressed as the coefficient of resistance as compared to the *Staphylococcus aureus* H standard. The standard organism was inhibited by 0.02 Oxford units per ml.

The resistance to penicillin of the strains under investigation is recorded in tables 1 and 4. It may be seen that, in general, the resistance of organisms of the *Streptococcus s b e* group to penicillin *in vitro* was found to be somewhat higher than that of the organisms of the unclassified group. However, highly resistant strains were not encountered, and all appeared within practical therapeutic range. Only the enterococci showed marked resistance to penicillin.

TABLE 4

*Penicillin resistance of nonhemolytic streptococci from subacute bacterial endocarditis*

PENICILLIN RESISTANCE (X STANDARD)	STREPTOCOCCUS S B E (9)	STREPTOCOCCUS SALIVARIUS (2)	ENTEROCOCCUS (2)	UNCLASSIFIED STREPTOCOCCI (21)
1	—	—	—	2
1.5	—	—	—	2
2	3	—	—	3
2.5	—	—	—	1
5	1	1	—	9
10	4	—	—	3
15	1	1	—	1
150	—	—	1	—
250	—	—	1	—
1-5	4	1	0	17
6-15	5	1	0	4
Above 16	0	0	2	0

It may also be noted from the tables that identification of *Streptococcus s b e* by this characteristic alone is not feasible.

#### SUMMARY

The serological and biological characteristics and the *in vitro* penicillin resistance of 34 strains of nonhemolytic streptococci isolated from 32 cases of subacute bacterial endocarditis were investigated.

The report of the existence of a new variety of nonhemolytic streptococcus, based upon serological and biological characteristics and designated as *Streptococcus s b e*, is confirmed.

The resistance of the *Streptococcus s b e* (*Streptococcus sanguis* White) group to penicillin was found to be slightly higher than the unclassified streptococci isolated from this disease, but none of the strains were markedly resistant to the antibiotic *in vitro*. The streptococci of the enterococcus group were found to be markedly resistant to penicillin.



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# THE *l*-AMINO ACID OXIDASE OF MOLDS<sup>1</sup>

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The role of amino acid oxidase (deaminase) in the metabolism of molds has been confined largely to a study of *d*-amino acid oxidase in *Neurospora* by Horowitz (1944). The *l*-amino acid oxidase of animal and bacterial cells has, however, been given considerable attention by Blanchard, Green, Nocito, and Ratner (1944), Stumpf and Green (1944), and Gale (1943). It is the purpose of this paper to report on the *l*-amino acid oxidase activity of penicillin-producing molds as well as of some other molds.

## EXPERIMENTAL METHODS

The molds were grown in shake flask fermentations according to the method that has been described by Koffler, Emerson, Perlman, and Burris (1945). Unless otherwise stated, the molds were grown in a medium made of 2 per cent corn steep liquor solids and 2 per cent crude lactose at pH 4.5.

The *l*-amino acid oxidase activity of the mold preparations was determined with the Warburg respirometer by measuring the oxygen uptake after adding 0.5 ml of the desired amino acid solution to the cell suspension. The ammonia that was produced by the deamination was determined by the micro method described by Umbreit, Burris, and Stauffer (1945).

The use of resting and washed cells either with or without "blending" (Dorrell and Knight, 1947) gave erratic results. Cells prepared in the following manner, however, gave consistent results. The mycelium was harvested by straining the contents of the shake flasks through cotton gauze and washing once with distilled water, the excess water was pressed out by hand. The damp mycelium was stirred quickly into acetone held at  $-20^{\circ}\text{C}$  in a dry-ice-acetone bath, after being stirred at  $-20^{\circ}\text{C}$  for 3 minutes the mycelium was washed once with acetone at  $4^{\circ}\text{C}$  and dried in a vacuum desiccator at room temperature. After drying, the mycelium was ground to a fine powder and stored at  $4^{\circ}\text{C}$ , where it remained active for at least 10 days. Usually the mycelium was harvested at 3 days of age since maximum oxidase activity was evident at that time (table 4).

A number of experiments on methods showed that the following experimental conditions gave reliable results. Maximum *l*-amino acid oxidase activity was obtained at pH 8.0 to 8.5 with either a  $\text{M}/15$  phosphate or borate buffer. At pH 7.0 and pH 6.0 the activity was decreased 20 and 50 per cent, respectively, the enzyme was almost inactive at pH 5.0. Fifty mg of dried and powdered mycelium were added to each Warburg flask although a straight-line relationship

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

existed between the amount of mycelium added (5 to 80 mg) and the amount of oxygen consumed, the rate of oxygen consumption remained constant for at

TABLE 1

Rate of oxidation of amino acids by l-amino acid oxidase in acetone dried mycelium of *P chrysogenum* NRRL 1951-B25

SUBSTRATE	OXYGEN UPTAKE	
	Minutes	
	80	160
	mm <sup>3</sup>	mm <sup>3</sup>
l-Alanine	58	113
dl-Alanine	49	90
dl-Alphaaminobutyric acid	46	90
dl-Alphaaminovaleric acid	37	75
dl-Alphaaminocaproic acid	28	51
dl-Valine	16	22
l-Methionine	53	111
dl-Methionine	50	94
l-Cystine	10	19
l-Glutamic	9	21
l-Aspartic	7	14
l-Proline	7	14
l-Leucine	4	5
l-Tryptophane	4	
l-Phenylalanine	2	
Glycine	3	5

Each Warburg flask contained 2.5 ml M/15 phosphate buffer at pH 8.0, 50 mg powdered mycelium, 0.2 ml 20 per cent KOH in the center well, and 0.5 ml M/10 l amino acid or 0.05 ml M/5 dl-amino acid in the side arm

TABLE 2

The relation between oxygen uptake and ammonia released by l amino acid oxidase in acetone dried mycelium of *P chrysogenum* NRRL 1951-B25 and Q176

MOLD	SUBSTRATE	OXYGEN UPTAKE	AMMONIA RELEASED
		microatoms	micromoles
NRRL 1951-B25	l-Alanine	11.7	12.3
	l-Methionine	19.0	18.4
Q176	l-Alanine	36.6	37.4
	l-Methionine	52.4	50.9

least 4 hours. Usually 0.5 ml of M/10 l-amino acid or 0.5 ml of M/5 dl-amino acid in M/15 phosphate buffer at pH 8.0 were added to each Warburg flask from the side arm after equilibration. This was considerably more amino acid than

was necessary to obtain maximum oxygen consumption during a 2-hour period. The temperature chosen for the determinations was 30 C, however, later it was found with the Thunberg technique and *l*-alanine as a substrate that the most rapid methylene blue reduction occurred at 50 to 55 C.

TABLE 3

*The effect of inhibitors on the l amino acid oxidase of acetone-dried mycelium of P chrysogenum NRRL 1951-B25 with l alanine as the substrate*

(Oxygen uptake was the criteria of enzyme activity)

INHIBITOR	CONCENTRATION ADDED PER FLASK	INHIBITION
	<i>moles</i>	<i>per cent</i>
Ammonium sulfate	0 1	77
	0 01	19
	0 001	0
Sodium sulfate	0 1	0
Copper sulfate	0 1	100
	0 01	76
Potassium cyanide	0 1	0
Sodium azide	0 1	0
2,4-Dinitrophenol	0 001	79
	0 0001	32
Capryl alcohol	Sat soln	100
Benzoic acid	0 05	53
	0 01	7
Sodium fluoride	0 1	0
Iodoacetic acid	0 1	76
	0 01	28
	0 001	0
Penicillin G	10 0 mg	0

## EXPERIMENTAL RESULTS

The amino acid oxidase (deaminases) of animal and bacterial origin oxidatively deaminate certain amino acids more rapidly than others. That the same is true apparently for the *l*-amino acid oxidase of *Penicillium chrysogenum* NRRL1951-B25 is shown in table 1. Slight glycine oxidase activity was apparent in this preparation as in most of the others. Since the presence of the *d*-isomers of alanine and methionine did not result in higher oxygen uptakes, the *d*-amino acid oxidase either was not present in the original tissue or was

destroyed by the drying procedure. The ability of the *d*-isomer of alanine and to a lesser extent of methionine to inhibit the *l*-amino acid oxidase of *P chrysogenum* NRRL 1951-B25 was evident also when the pure *d*-isomer was added with the *l*-isomer. The short chain monoamino, monocarboxyl amino

TABLE 4

*A comparison of the l-amino acid oxidase activity of the acetone dried mycelium of various molds on three l-amino acids*

(Oxygen uptake in 80 minutes was the measure of oxidase activity)

MOLD	SUBSTRATE	OXYGEN UPTAKE		
		Age of mycelium at harvest (days)		
		3	5	7
		mm <sup>3</sup>	mm <sup>3</sup>	mm <sup>3</sup>
Penicillin producers				
<i>P notatum</i> 832	<i>l</i> -Alanine	29	13	6
	<i>l</i> -Phenylalanine	0	0	
<i>P chrysogenum</i> NRRL 1951-B25	<i>l</i> -Alanine	55	40	19
	<i>l</i> -Methionine	48	33	14
	<i>l</i> -Phenylalanine	3	0	
X1612	<i>l</i> -Alanine	63	50	39
	<i>l</i> -Methionine	63	54	38
	<i>l</i> -Phenylalanine	5	2	
Q176	<i>l</i> -Alanine	115	88	62
	<i>l</i> -Phenylalanine	11	4	4
Non-penicillin-producers				
<i>P expansum</i>	<i>l</i> -Alanine	95	71	
	<i>l</i> -Methionine	80	55	
	<i>l</i> -Phenylalanine	11		
<i>P sanguineum</i>	<i>l</i> -Alanine	21	7	
	<i>l</i> -Methionine	22	12	
	<i>l</i> -Phenylalanine	12	4	
<i>Aspergillus niger</i>	<i>l</i> -Alanine	19	13	
	<i>l</i> -Methionine	21		
	<i>l</i> -Phenylalanine	14	7	
<i>P notatum</i> 174*	<i>l</i> -Alanine	31		8

\* A non penicillin producing mutant of *P notatum* 832

acids were much more susceptible to the amino acid oxidase than were the longer, more complex amino acids

Table 2 shows that 1 atom of oxygen was taken up for each molecule of ammonia that was liberated. Since the preparation contained catalase, it was not

necessary to add any other source of this enzyme. On the basis of these data it seemed reasonable to assume that the *l*-amino acid oxidase of *P. chrysogenum* NRRL 1951-B25 and Q176 followed the usual over-all equation



The effect of inhibitors on the *l*-amino acid oxidase activity of strain NRRL 1951-B25 is summarized in table 3. The *l*-oxidase from preparations of strain Q176 followed the same pattern and was inhibited by ammonium sulfate, 2,4-dinitrophenol, capryl alcohol, and benzoic acid. Although *l*-alanine was the substrate for all inhibitor tests, there was no reason to suspect that the inhibitors would react differently with different substrates.

Table 4 compares the *l*-oxidase activity of different molds grown in submerged culture and harvested at 3, 5, and 7 days of age. The molds with the highest

TABLE 5

*The influence of the nitrogen source on the l-amino acid oxidase of acetone dried mycelium of P. chrysogenum NRRL 1951-B25*

(Oxygen uptake in 80 minutes was the measure of oxidase activity)

MEDIUM	N SOURCE	OXYGEN UPTAKE
		mm <sup>3</sup>
I*	Corn steep solids	43
I plus 0.5% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Corn steep solids plus (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	9
II† plus 1.0% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5
II plus 0.5% corn steep solids	Corn steep solids	48
II plus 0.5% peptone	Peptone	36
II plus 1.0% <i>dl</i> -alanine	<i>dl</i> -Alanine	39

\* I, corn steep liquor lactose medium

† II, synthetic base: KH<sub>2</sub>PO<sub>4</sub>, 2.5 g; MgSO<sub>4</sub>, 0.5; FeSO<sub>4</sub>, 0.05; ZnSO<sub>4</sub>, 0.01; glucose, 10.0; distilled water, 1 liter. N source as indicated in table.

penicillin-producing potential had the highest oxidase content, however, the non-penicillin-producing molds also produced the enzyme. Maximum *l*-amino acid oxidase activity was evident in mycelium that was harvested at 3 days of age; mycelium that was harvested at 5 and 7 days had less activity. All of the molds grew well in the corn steep liquor lactose medium. Surface-grown cultures of strain NRRL 1951-B25 and *Penicillium expansum* had as much oxidase activity as submerged cultures. Attempts to secure *l*-amino acid oxidase activity from *Neurospora crassa* by the acetone drying procedure were unsuccessful.

The composition of the medium had a great influence on the *l*-amino acid oxidase of strain NRRL 1951-B25. Table 5 shows that maximum oxidase activity was obtained when the mold was grown on corn steep liquor lactose medium or in a synthetic basal medium with either *dl*-alanine, peptone, or corn steep liquor as the nitrogen source. Oxidase activity was low when the mold was grown in the synthetic basal medium with ammonium sulfate as the nitrogen source. The activity was decreased considerably also when ammonium sulfate

was added to the corn steep liquor medium. Extremes in pH were not responsible for the oxidase content of the mycelia since the range of pH at the time of harvest was 4.5 to 5.2.

Methylene blue (Thunberg technique) could replace oxygen as the hydrogen acceptor, but the reaction was sluggish. Nevertheless, the Thunberg technique was used to determine the temperature range of the enzyme.

Numerous attempts were made to obtain an active, cell-free preparation from strain NRRL 1951-B25, but the enzyme seemed to adhere closely to the particulate matter. Recently, however, a cell-free preparation that contained practically all the enzyme has been prepared from strain Q176. The cell-free preparation is free of glycine oxidase, otherwise it checks with the data reported for acetone-dried cells of Q176.

#### DISCUSSION

The *l*-amino acid oxidase from the molds that were tested was different from the oxidase of rat kidney and *Proteus vulgaris* in certain respects. One of the most striking differences was that the mold oxidase deaminated the simple short chain amino acids more rapidly than the longer, branched, or substituted acids. This is exactly the opposite of the rat and *Proteus* oxidases. Nevertheless, the three oxidases seem to be similar in that 1 atom of oxygen was taken up for each molecule of ammonia that was released in the catalyzed reaction between oxygen and the *l*-amino acid. All three enzymes differed somewhat in their response to inhibitors. Except for its sensitivity to capryl alcohol, the oxidase from mold behaved much like the oxidase from rat kidney. The three enzymes were sensitive to copper sulfate.

Contrary to the behavior of other *l*-amino acid oxidases that have been described, the acetone-dried preparations of molds were sluggish when tested with the Thunberg technique. This failure to use methylene blue readily as a hydrogen acceptor is in keeping with the aerobic nature of the molds.

In contrast to the *Escherichia coli* deaminases that have been described by Gale (1943), the mold deaminases were produced at a low pH during active growth and not at an alkaline pH during the final stages of growth. It is of interest that the oxidase had maximum activity at about pH 8.0, yet was produced by the mold while it was growing at pH 4.0 to 5.0. The oxidase content of *P. chrysogenum* NRRL 1951-B25 and Q176 dropped after the fourth day of growth, and at 7 days (pH 7.2 to 7.8) less than one-half of the original activity remained. This is in accord with the fact that the same molds have a greatly decreased  $Q_{O_2}$  during the phase of penicillin production, i.e., 6 to 8 days (unpublished data). On the basis of these observations plus those formerly reported on the chemical changes during penicillin production (Koffler, Knight, Emerson, and Burris, 1945) it can be concluded that penicillin is produced by the mold when it has passed the peak of metabolic activity and is approaching autolysis.

It is interesting that the *l*-amino acid oxidase content of the penicillin-producing molds can be correlated with the penicillin-producing ability of the mold. Strain 832, one of the early penicillin-producing molds, had a low oxidase ac-



tivity, a higher penicillin producer, NRRL 1951-B25, had higher oxidase activity, and so on through X1612 and Q176. Somewhat the same condition exists with regard to the endogenous  $Q_{O_2}$  values of penicillin-producing molds 832, NRRL 1951-B25, X1612, and Q176, the highest penicillin producers have the highest  $Q_{O_2}$  values (unpublished data). It is difficult to draw any conclusions from this information except that the highest penicillin producers are metabolically more active than the low penicillin producers.

The fact that the most deaminase was produced when the mold was forced to obtain its nitrogen from alanine, peptone, or corn steep liquor solids suggests that ammonia may be the preferred nitrogen source. The major role of the oxidative deaminases in mold physiology may be to liberate ammonia from amino acids in the medium. Current research is designed to test this theory.

#### ACKNOWLEDGMENT

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#### SUMMARY

A method for demonstrating *l*-amino acid oxidase in molds has been described. The enzyme was found in a number of molds at 3 to 7 days of age. The amount of *l*-amino acid oxidase in the mold depended upon the nitrogen source and the age of the mycelium.

The *l*-amino acid oxidase of molds differed in some respects from the oxidase of rat kidney and *Proteus vulgaris*. The oxidases were similar in that one atom of oxygen displaced one molecule of ammonia in the catalyzed reaction between the *l*-amino acid and oxygen.

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# SOME PROPERTIES OF AN ANTIBIOTIC OBTAINED FROM A SPECIES OF STREPTOMYCES<sup>1</sup>

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Despite intensive research during the past 7 years, only a few antibiotics are in general therapeutic use. Many others have been described that are effective against the gram-positive bacteria but only a very few have been reported effective against the gram-negative bacteria which at the same time are nontoxic to higher animals. In a search of the actinomycetes for antibiotic-producing organisms a new and hitherto-undescribed antibiotic<sup>4</sup> has been obtained and extracted in a crystalline form. The antibacterial spectrum and chemical properties differentiate it from other materials, and preliminary studies indicate that this material has a low order of toxicity.

## METHODS

Initial isolations of antibiotic-producing cultures from soil suspensions were made on an asparagine agar medium, and the colonies were then transferred to test tubes containing a modified Emerson's agar. After the test tube culture had grown for 2 weeks, pieces of medium were removed from the periphery of the mycelium and placed on four different plates, each one seeded with one of the following organisms: *Bacillus subtilis*, *Aerobacter aerogenes*, *Sclerotinia fructicola*, and *Fusarium lycopersici*. These seeded plates were observed for inhibition zones, and only those cultures that inhibited the growth of *Aerobacter aerogenes* or the fungi were retained for study. Further tests of antibiotic activity were made by streaking the actinomycetes on Emerson's agar in petri plates and counter-streaking bacteria perpendicularly to the actinomycete after 10 days. The ability of an actinomycete to produce the antibiotic in various liquid media was determined on 125 ml of broth in a 500-ml flask on a reciprocal shaker. Assays to determine relative concentrations in terms of streptomycin units and also assays of the crystalline compound were made by the disk method as described by Loo *et al.* (1945).

## THE ORGANISM

Isolate 8-44 was one of those obtained from a compost on the South Farm of the University of Illinois at Urbana, Illinois. It belongs to the genus *Strepto-*

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<sup>4</sup> After the preparation of this manuscript, a paper appeared describing a similar antibiotic from an actinomycete obtained from South America (Ehrlich *et al.*, 1947).

*myces* as described by Waksman and Henrici (1943) The organism forms heavy wrinkled mycelium that on Emerson's medium has a light yellow back. Good sporulation occurs within 7 to 10 days on Emerson's medium, and the spore surface of the culture presents a white to light pink appearance. Various other colors are produced in different media. The organism is gram-positive and has aerial spores.

#### BACTERIAL SPECTRUM

Isolate 8-44 inhibited a wide range of organisms when grown in Emerson's medium. Antibiotic activity has been observed against 22 different bacteria of the gram-negative and gram-positive groups, including some acid-fast bacteria.

TABLE 1

*Comparative spectra of 8-44 and other antibiotic-producing actinomycetes*

BACTERIA	ZONE OF INHIBITION IN MM FROM STREAK OF				
	<i>S. griseus</i>	<i>S. antibioticus</i>	<i>S. lavendulae</i>	<i>N. gardnerii</i>	8-44
<i>P. aeruginosa</i>	18	0	18	0	0
<i>A. aerogenes</i>	>45	0	>45	0	745
<i>A. faecalis</i>	>45	0	>45	0	>45
<i>B. subtilis</i> (Ill)	37	>45	>45	>45	41
<i>B. subtilis</i> ('mycin res')	10	30	>45	41	>45
<i>B. subtilis</i>	35	>45	28	33	>45
<i>S. faecalis</i>	21	27	6	>45	>45
<i>S. lactis</i>	17	33	8	38	>45
<i>M. smegmatis</i>	35	25	38	0	37
<i>E. coli</i>	25	0	30	0	17
<i>S. aureus</i>	>45	18	35	>45	>45
<i>M. flavus</i>	38	30	39	—	>45

Only one organism, *Pseudomonas aeruginosa*, was not inhibited in any of the tests.

The growths of the following bacteria were inhibited either on agar streak plates or in broth culture: *Escherichia coli*, *Aerobacter aerogenes*, *Salmonella pullorum*, *Pasteurella multocoides*, *Salmonella schottmuelleri*, *Shigella paradysenteriae* (Sonne), *Serratia marcescens*, *Eberthella typhosa*, *Proteus vulgaris*, *Brucella abortus*, *Agrobacterium tumefaciens*, *Streptococcus hemolyticus*, *Streptococcus lactis*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Alcaligenes faecalis*, *Bacillus globigii*, *Bacillus subtilis*, *Micrococcus flavus*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* var *hominis* (avirulent).

Comparative spectra were made with a number of antibiotic-producing actinomycetes to ascertain the singularity of the active material produced by 8-44. An examination of table 1 reveals that the substance produced by 8-44 can be differentiated from streptomycin by the absence of activity against *Pseudomonas aeruginosa* and by its inhibition of a streptomycin-resistant strain of *Bacillus*.

*subtilis* It differs from streptothricin, since 8-44 is very effective against *Streptococcus lactis* and *Streptococcus faecalis*, but inactive against *Pseudomonas aeruginosa*, the converse occurs with streptothricin Protoactinomycin from *Nocardia gardneri* is inactive against *Aerobacter aerogenes*, *Alcaligenes faecalis*, *Escherichia coli*, and *Mycobacterium smegmatis*, whereas all these organisms are inhibited by 8-44 Similarly the first three of these bacteria are not inhibited by *Streptomyces antibioticus*, which differentiates actinomycin from the product of 8-44 A survey of the literature indicates that the new antibiotic is also different from actinomycetin, bacitracin, subtilin, and micromonosporin since these four substances are not primarily active against the gram-negative bacteria

#### GROWTH AND ANTIBIOTIC PRODUCTION IN SHAKE FLASKS

Isolate 8-44 grew readily in a number of liquid media on a reciprocal shaker, but antibiotic production was more limited The organism grew slowly in Emerson's medium,<sup>5</sup> more rapidly in a corn steep medium,<sup>6</sup> and best in a soy-

TABLE 2  
*Antibiotic activity of 8-44 in shake flasks*

EXPERIMENT	STREPTOMYCIN UNITS AT VARIOUS TIMES IN HOURS							
	24	48	72	96	120	144	168	192
22	—	108	108	260	207	260	74	—
28	—	31	48	108	74	74	205	—
29	50	89	108	74	167	260	—	—
32	—	—	—	74	144	108	108	108

bean medium,<sup>7</sup> however, it produced the antibiotic only in the soybean medium Shake cultures were inoculated with the spores and mycelium of a 10-day-old test tube culture After 5 days on the shaker, 3-ml aliquots of these cultures were added to 125 ml of the media contained in 500-ml Erlenmeyer flasks Triplicate flasks were used for all assays, and results were reported in terms of equivalent streptomycin units As indicated in table 2, values equivalent to 260 streptomycin units were obtained in the brews The maximum production was usually obtained between the fourth to seventh day after inoculation, and as evaluated on the basis of subsequent assays on crystalline preparations these liquors contained as much as 0.08 mg per ml

The media from 5-day-old shake flask cultures were examined to determine whether the same antibiotic activity was produced under these conditions as

<sup>5</sup> *Emerson's medium* beef extract, 4.0 g, peptone, 4.0 g, NaCl, 2.5 g, yeast extract, 1.0 g, glucose, 10.0 g, distilled water, 1,000 ml

<sup>6</sup> *Corn steep* peptone, 5.0 g, corn steep, 15.0 g, NaCl, 5.0 g, glucose, 10.0 g, distilled water, 1,000 ml

<sup>7</sup> *Soybean* soybean meal, 10.0 g, cerelese, 10.0 g, NaCl, 5.0 g, curbay B G, 0.5 g, CaCO<sub>3</sub>, 1.0 g, distilled water, 1,000 ml

from streaks of 8-44. A similar spectrum of inhibition was obtained from the shake culture as with the streaks. Growth of *P. aeruginosa* was not inhibited, whereas the following bacteria were *Alcaligenes faecalis*, *Aerobacter aerogenes*, *Salmonella pullorum*, *Streptococcus faecalis*, *Mycobacterium tuberculosis* (avirulent). When sterile filtrates of 8-44 and *Streptomyces griseus* were compared in agar dilution plates, the dilutions of 8-44 media inhibiting the bacteria were either the same as or lower than those of *S. griseus*. Antibiotic activity was obtained with 1/20 dilutions of the 8-44 liquor.

#### PHAGE

Because the occurrence of phage in cultures of *Streptomyces griseus* has been reported, the reaction of 8-44 to this phage was tested. Tests by Dr. D. R. Colingsworth of the Upjohn Company reveal that 8-44 is susceptible to their *S. griseus* phage.

#### ISOLATION AND CHEMICAL PROPERTIES OF THE ANTIBACTERIAL SUBSTANCE

The antibacterial activity of culture fluid was found to be stable at room temperature at pH 2 to 9 and was not destroyed by sterilization in the autoclave at 110°C at pH 4 to 8. The active material could be removed from the culture fluid on charcoal or alumina and was readily extracted with butanol or ether. For preparative purposes the culture medium was extracted with an equal volume of ether. The ether extract was dried and the ether removed leaving a brown, gummy, semicrystalline residue that assayed 90 to 100 streptomycin units per mg and accounted for 80 to 90 per cent of the original activity. This material was extracted with hot benzene (50 ml per 0.2 g), which removed the gummy material, leaving a yellow crystalline solid that assayed 300 to 500 streptomycin units per mg. A small additional amount of the same solid separated from the benzene extract on cooling. This crude material was recrystallized from a benzene-methanol mixture and was then chromatographed over alumina in 20 volumes of a chloroform-methanol mixture (30 per cent methanol). The light yellow eluate was concentrated to dryness, and the residue was recrystallized from chloroform-methanol mixture (5 per cent methanol) giving fine needle-shaped crystals melting at 144 to 146°C. Repeated crystallization gave an almost white product.

With 7 liters of culture fluid assaying 28 streptomycin units per ml the yield of purified material was 0.20 g, assaying 630 streptomycin units per mg. This represents an over-all recovery of 62 per cent.

The purified material was insoluble in petroleum ether, slightly soluble in chloroform, and soluble in ether, methanol, ethanol, or ethyl acetate. It was very sparingly soluble in water, acid, alkali, or hot benzene. The final product contained nitrogen and halogen, but no sulfur, and gave negative Sakaguchi and Pauly tests.

Chemical work on the active substance was discontinued at this point since it was learned that the Parke, Davis group had already elucidated the structure

of this compound, for which they have proposed the name chloromycetin. The following comparison of our material and the Parke, Davis product was made in the Parke, Davis laboratories through the courtesy of Dr. L. A. Sweet:

	Parke, Davis product	Illinois product
Melting point	149-150° C	144-147° C
Mixed melting point		148-149° C
Peaks of ultraviolet absorption spectra		
Water	278 m $\mu$	278 m $\mu$
0.1 N hydrochloric acid	278 m $\mu$	278 m $\mu$
0.1 N sodium hydroxide	279 m $\mu$	279.5 m $\mu$

These data in conjunction with the solubility properties and the antibacterial spectra leave no doubt as to the identity of the two substances.

#### ANTIBIOTIC ACTIVITY OF CHEMICAL PREPARATION

All studies of antibiotic activity of the active fraction were made with a "crude prep." Because of its low solubility in water, a stock solution was prepared containing 0.1 mg solid in 10 per cent ethyl alcohol and sterilized through a glass filter to prevent adsorption. Various dilutions in sterile, distilled water were made from the stock and added to double-strength Emerson's agar to give concentrations ranging from 1 part in 2,000 to 1 part in 20,000,000. The plates were held at 37° C for 24 hours before being streaked with the bacteria. That the crude preparation was the active principle originally in the culture medium was proved by its spectrum. All bacteria that were inhibited by the organism were also inhibited by the "prep." *P. aeruginosa*, which was not inhibited on streak plates, required a large concentration of the compound for inhibition, between 1 part in 2,000 and 1 part in 10,000. Generally the activity of this antibiotic compares favorably with the activity of "crude preps" reported in the literature for streptomycin and streptothricin. Most of the bacteria were inhibited at concentrations between 1 part in 200,000 and 1 part in 1,000,000. Substance 8-44 was particularly effective on a single isolate of *S. hemolyticus* and one of *B. abortus*, which were inhibited at concentrations of 1 part in 2,000,000 to 1 part in 10,000,000 (table 3). The activity of 8-44 was also tested in nutrient broth against two organisms obtained from the Food and Drug Administration, *E. coli* PC1-512 and *B. subtilis* PC1-220. Narrower concentration ranges were used in this experiment. *E. coli* was inhibited at about 1 part in 1,000,000 and *B. subtilis* at about 1 part in 500,000. *Salmonella schottmuelleri* and *Shigella paradysenteriae* (Sonne) were tested in a Difco brain heart infusion, and approximately 50 per cent inhibition was obtained at 0.50  $\mu$ g per ml and 0.25  $\mu$ g per ml, respectively.

#### ASSAY PROCEDURES

Difficulties were encountered in assigning unit values to the antibiotic activity of shake cultures when interpreted as streptomycin units. All assays were run

by the paper-disk plate method, and a phosphate buffer was used to make the dilutions. When such dilutions were made, the values invariably were lower than expected. Thus if an undiluted sample gave 432 units, the same sample in a 1:3 dilution gave 37 units. If the undiluted value was divided by 4 to make the samples comparable, the result would be 108 and 37 units, respectively, from the same sample. Therefore, experiments were made to determine the

TABLE 3  
*Inhibition of various bacteria by antibiotic 8-44 in agar*

ORGANISM	MINIMUM INHIB CONC μG/ML
	<i>complete inhibition in agar</i>
<i>P. aeruginosa</i>	100-500
<i>Alcaligenes faecalis</i>	50-100
<i>E. coli</i>	10-50
<i>Mycobacterium smegmatis</i>	10-50
<i>M. tuberculosis</i> var <i>hominis</i> (avirulent)	10-50
<i>B. globigii</i>	10-50
<i>Aerobacter aerogenes</i>	1-5
<i>B. subtilis</i>	"
<i>B. subtilis</i> (streptomycin-resistant)	"
<i>B. subtilis</i> (streptothricin-resistant)	"
<i>S. aureus</i>	"
<i>A. tumefaciens</i>	"
<i>S. faecalis</i>	"
<i>S. lactis</i>	"
<i>S. hemolyticus</i> HH6 (Ill Dept of Bact)	"
+ " HH13	"
" HH20	"
" HH7	0.1-0.5
<i>Brucella abortus</i>	"
	<i>50% inhibition in broth*</i>
<i>Salmonella schottmuelleri</i>	0.50
<i>Shigella paradysenteriae</i> (Sonne)	0.25

\* These determinations were made at the Parke, Davis Research Laboratories through the courtesy of Dr. John Ehrlich. They represent concentrations giving 50 per cent inhibition when read turbidimetrically from broth dilution cultures (Joslyn and Galbraith, 1947).

effect of dilution upon ring size with 8-44 and streptomycin. It was found that ring size is cut down more sharply with dilution of 8-44 than with streptomycin broths, and thus gives lower values when interpreted as streptomycin units. A different type of relation must then exist between the concentration of 8-44 and ring size, compared to that of streptomycin. Further studies were conducted on assay procedures to compare the inhibition zone of *B. subtilis* with various concentrations of crystalline 8-44 and high purity streptomycin. The standard curves were established with concentrations of 0.01 mg per ml to 0.2 mg per ml.



The 8-44 was dissolved in 10 per cent ethyl alcohol to allow for greater solubility, and the streptomycin was dissolved in the regular phosphate buffer. The average zones of inhibition from six replicates were plotted against concentration. A typical curve is shown in figure 1. The streptomycin and 8-44 curves cross each other at a concentration of 0.08 mg per ml. At lower concentrations streptomycin gives larger zones of inhibition, and at higher concentrations 8-44 gives greater inhibition. The difference in these curves can be used to explain the low values obtained when 8-44 is interpreted in streptomycin units, and it is obvious that the more sensitive strains of *B. subtilis* that are usually used for assaying at lower potencies of streptomycin would show larger discrepancies. A straight line is obtained when the 8-44 assay values are plotted on log paper.

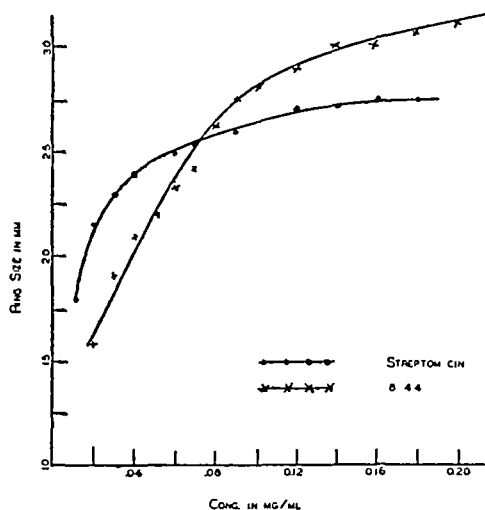


FIG 1 RELATION BETWEEN CONCENTRATION AND RING SIZE

between concentrations of 0.02 mg per ml and 0.12 mg per ml, beyond that the slope of the curve decreases.

The edge of the inhibition zone produced by 8-44 is not so sharp as that produced by streptomycin. Attempts were made to sharpen the ring by two methods: (1) varying the pH of the antibiotic medium and also of the sugar medium between pH 5.7 and 8.0, and (2) using test organisms other than *B. subtilis*. With neither method was the desired end obtained. The rings can best be measured with rulings etched into glass and viewed against a black background. Some preliminary experiments indicate the possibility of a turbidimetric assay procedure for 8-44.

#### TOXICITY

Preliminary studies on the culture filtrate of 8-44 showed the absence of any substances toxic to guinea pigs when it was injected subcutaneously at the rate

of 10 ml sterile broth per kilogram of body weight<sup>8</sup> The crystalline preparations of 8-44 also showed no deaths of mice when injected at a concentration of 10 mg per 20-gram mouse in 0.4 ml of 50 per cent propylene glycol<sup>9</sup>

#### DISCUSSION

The discovery of more antibiotics capable of attacking the gram-negative bacteria is important not only because they might be efficient for diseases relatively difficult to control with streptomycin, but also because of the ease with which many bacterial populations develop resistance to this antibiotic. Though the therapeutic value of 8-44 is unknown, it inhibits a wide variety of bacteria and deserves further investigation. It is capable, in at least the case of *B. subtilis*, of *in vitro* inhibition of bacteria that have acquired resistance to streptomycin. Compound 8-44 possesses other qualities that might be advantageous for therapeutic use. It is very stable to both acids and alkalis and thus, if absorbed by the enteral tract, might be administered orally. The relative insolubility in water might not be a limiting factor with this type of administration. For subcutaneous injections the solubility of 8-44 in propylene glycol provides a means of more direct entrance into the body. Another advantage of 8-44 is the ease with which it can be extracted and purified. One feature of the organism, 8-44, that requires more study is its susceptibility to phage, and efforts should therefore be made to isolate and train toward phage-resistant strains to forestall the limiting action of such viruses on the production of 8-44.

We contemplate no further investigations of 8-44, since upon the completion of this phase of our studies we have learned that the same compound has been independently isolated at the Parke, Davis Research Laboratories and a publication from that institution is now in press.

#### ACKNOWLEDGMENT

The writers wish to express their appreciation to Mrs. Betty Johnpeter for her assistance in the biological phases of this investigation.

#### SUMMARY

An antibiotic has been described which, *in vitro*, inhibits at least 22 different bacteria of the gram-negative, gram-positive, and acid-fast groups. It is produced by a *Streptomyces* and is readily extracted from the growth medium. The organism is readily attacked by a *Streptomyces griseus* phage. The crystalline substance is relatively insoluble in water but soluble in some organic solvents. It is stable to heat, to acid, and to alkali, and is readily adsorbed from solution by activated charcoal and other agents. A comparison of certain physical and chemical properties of this substance and chloromycetin has shown the two to be identical.

<sup>8</sup> These tests were made by Dr. N. Levine of the College of Veterinary Medicine of the University of Illinois.

<sup>9</sup> Toxicity tests with the crystalline material were made available through the courtesy of Dr. George F. Cartland of the Upjohn Company.

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## THE ISOLATION OF SALMONELLA TYPHI-MURIUM FROM FERRETS

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Ferrets for use as test animals in a series of studies of a viral agent were purchased from an outside source. Animals so obtained for use at this laboratory are held for an observation period of 3 weeks, a time which exceeds the incubation period of the viral infection.

At the expiration of the observation period for one shipment of ferrets three animals of the lot were inoculated with virus from the same source. One of the three animals was sacrificed for serum the day following inoculation, and the organs were not cultured. The livers and spleens of the remaining two animals were cultured, at the time of tissue harvest, on blood agar. The organs from one of the animals gave rise to no growth. A gram-negative rod in pure culture was isolated from the other. The spleen of the infected animal was triturated and inoculated into two ferrets. The temperature curves of these two animals were not characteristic of the type commonly found to result from the viral agent used. Ten days following inoculation one of the animals was moribund. Both were sacrificed.

Post-mortem examination showed emaciation of both animals. Both exhibited conjunctivitis with a clear watery discharge. In one, balanitis with a purulent exudate was present. A dark fibrinous exudate in the lower intestine of one animal was matched in the other by a tarry content throughout the intestinal tract. Petechial hemorrhages in the gastric mucosa indicated the probable source of the digested blood in the latter animal. Except for a marked anemic condition, other visceral organs appeared normal. These findings, suggestive of paratyphoid infection, necessarily represented a subacute rather than a chronic form of the disease as commonly observed in related species of animals. Symptoms of emaciation, conjunctivitis, and balanitis indicated the probability of infection prior to artificial inoculation.

Bacteriological cultures of livers and spleens were prepared on blood agar. Cultures of *Salmonella typhi-murium* were obtained from both animals without interference of contaminants.

The organism isolated was a gram-negative, motile, aerobic rod. It produced no indole when grown in tryptone broth. Nitrates were reduced to nitrites.

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H<sub>2</sub>S was produced when the organism was cultivated on Kligler iron agar. It reacted negatively to the Voges-Proskauer test and positively to the methyl red test.

Acid and gas were formed from glucose, mannitol, fructose, arabinose, xylose, dulcitol, inositol, trehalose, and maltose. Lactose and sucrose were not attacked. The antigenic formula was determined as IV, V, XII, 1, 1, 2, 3.<sup>3</sup>

Subcultures of the organisms were inoculated into a ferret and into mice. A 24-hour plate culture suspended in 10.0 ml physiological saline was inoculated subcutaneously in 0.2-ml amounts into each of five mice. The ferret received 1.0 ml of this suspension subcutaneously. All mice were dead at the end of 18 hours. The ferret was found dead at 36 hours. The most pronounced lesion in the inoculated ferret was a suppurating ulcer at the site of inoculation. Pure cultures of *S. typhi-murium* were recovered from the spleen and liver. No attempt was made to recover the organism from the inoculated mice.

So far as we are aware paratyphoid infection has not been previously described in ferrets. Dr P. R. Edwards of the National Salmonella Center, in a recent communication, states that he has no record of any *Salmonella* being isolated from this species.

#### SUMMARY

The isolation of *Salmonella typhi-murium* from ferrets is recorded. The symptoms and pathologic findings associated with paratyphoid infection are given, and the isolated organism is described. So far as we are aware no *Salmonella* has previously been described from ferrets.

<sup>3</sup> The antigenic formula was determined by the Division of Bacteriology, Army Medical Department Research and Graduate School.

# FACTORS AFFECTING THE PRODUCTION OF TETANUS TOXIN TEMPERATURE<sup>1</sup>

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The writers have reported (Mueller and Miller, 1947) the production of a highly potent toxin by what appears to be a variant strain of *Clostridium tetani*. The medium that is employed contains beef heart infusion and a tryptic digest of casein, and an excess of iron. Studies have been in progress for some time in an attempt to define the chemical nature of the essential components of this medium. Certain advances have been made in this direction that will be reported later. The present communication deals with a relatively minor technical detail, yet one found to have so striking an influence on toxin yield that it seems worth brief mention: the temperature of incubation, a matter usually receiving reasonable attention, appears in this case to deserve exceptionally careful control.

Over a considerable time periodic difficulty has been experienced in obtaining the expected yield of 60 to 70 Lf of toxin in control tubes included in experiments. When this occurs, the significance of the whole experiment is open to question. Various points in technique have been explored in an effort to avoid this difficulty, but usually the matter straightened itself out for no very obvious reason. Eventually attention was directed to the temperature of incubation. Cultures presumably grew for 5 days at 34 to 35 C, but it appeared that in hot weather or in exceptionally cold periods there might be variation above or below these figures. Preliminary trials in which tubes were incubated in a constant temperature water bath indicated a temperature effect so striking that a careful series of experiments was carried out in triplicate.

Water baths of the Warburg type were employed, in which temperatures remained constant to within  $\pm 0.1$  C. Medium prepared according to the formula previously described (Mueller and Miller, 1947) was distributed in 6-by- $\frac{3}{4}$ -inch tubes, a few mg of reduced Fe were added, and the tubes sterilized in flowing steam for 20 minutes. They were then cooled promptly in water and inoculated with a few drops of a 24-hour broth culture of *C. tetani*. Three tubes of medium were incubated in each of several water baths at different temperatures. It was thus possible to hold to a single variable and to obtain triplicate values at three to five different temperatures in each experiment. Table 1 presents the summarized results of three separate experiments.

Although the averages of the titers obtained at various temperatures do not fall on a smooth curve, they do indicate a definite and rather sharp optimum in the neighborhood of 35 C. Since this conclusion has been reached, experiments relating to the composition of the medium have been made with incubation in a

<sup>1</sup> Aided by a grant from the Commonwealth Fund

Warburg bath at 35.1 C. The titers of control tubes in 24 consecutive experiments so conducted are contrasted in table 2 with titers of the last 24 experiments previously performed in an ordinary bacteriological incubator. The thermostat of

TABLE 1  
*Lf of toxin produced in 5 days at various temperatures*

	TEMPERATURE (C)							
	32.1	33.6	34.3	35.1	35.7	36.2	37.1	38.2
Exp I—Lf/ml	—	—	85	—	—	65	80	—
	—	—	80	—	—	70	75	—
	—	—	70	—	—	70	70	—
Exp II—Lf/ml	35	—	60	95	—	—	75	20
	35	—	80	100	—	—	75	20
	35	—	85	98	—	—	65	17
Exp III—Lf/ml	—	85	—	110	105	—	—	—
	—	60	—	105	100	—	—	—
	—	90	—	110	65	—	—	—
Average Lf/ml	35	78	77	103	90	68	73	19

TABLE 2  
*Lf range of toxin in incubator and water bath*

	TUBES IN INCUBATOR	TUBES IN WATER BATH
<i>Lf/ml</i>		
20-29	2	—
30-39	2	—
40-49	4	—
50-59	9	—
60-69	5	—
70-79	2	2
80-89	—	3
90-99	—	8
100-110	—	11
Total	24	24

the latter was adjusted to 35 C, but the temperature surely varied by at least +1 C and may have gone to 37 C or above on hot days.

#### DISCUSSION

In general, the influence of temperature on bacterial growth is too well known to require extensive comment. It is thoroughly discussed in its various aspects by Porter (1946), and Dorn and Rahn (1939) have shown that even for the same organism different functions may require different optimal temperatures. For an



formation, influenced probably by many variables, is perhaps delayed by sub-optimal temperatures, whereas its denaturation may be accelerated by a moderate excess of heat. On the other hand, it is possible that as the resultant of multiple metabolic functions of the organism, any lack of balance in the entire growth process would be reflected in an alteration in the yield. It is probably significant that, although the incubator and water bath settings were practically identical, the former undoubtedly underwent periodic variations above and below 35 C, whereas in the water bath a considerable degree of uniformity was obtained. With this more careful control of temperature, a noteworthy improvement in regularity of toxin production was achieved, and average titers rose from 50 to 60 to 90 to 100 Lf per ml.

One of the puzzling difficulties encountered in our earlier work has been the failure to obtain as good yields of tetanus toxin in flasks or bottles of large size as in test tubes containing 20 ml of medium. Possibly these observations on temperature will offer a partial explanation for this phenomenon, although preliminary experiments indicate that it is not the only, or perhaps the most important, factor.

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*Turbidimetric test* Before the pure substance was obtained, a method of following antibacterial activity was employed in which the end point was considered to be that dilution of the antibiotic culture filtrate or concentrate that inhibited the growth of an actively growing test organism in broth by 50 per cent (Joslyn and Galbraith, 1947)

*Turbidimetric assay of chloromycetin* Methods for isolation of chloromycetin from culture liquids and certain physical and chemical properties of the crystalline substance have already been reported (Bartz, 1948) With the pure compound available,<sup>2</sup> it became possible to express the antibacterial activity of solutions of the antibiotic (culture liquids, body fluids, etc.) gravimetrically in terms of a crystalline reference standard A daily standard curve is drawn by plotting the percentage of growth of *Shigella paradysenteriae* (Sonne) on a linear scale against broth dilution of the standard on a logarithmic scale Samples for assay, in the form of clear but not necessarily sterile solutions, are diluted on the basis of estimated potency and their assay potency is calculated from the standard curve A more detailed description of the turbidimetric method of assay is in preparation

#### *Broth Culture Filtrates*

*Antibacterial activity* The organism was first grown in media 1 to 8, without agar, in 100-ml quantities in 500-ml Erlenmeyer flasks on a rotary shaking machine After an appropriate growth period the culture liquid was withdrawn, filtered to remove the organism, and tested The data on a typical early experiment are given in table 2 The antibiotic titer was relatively low The greatest dilutions of shaken culture filtrate giving 50 per cent inhibition of the test species were not determined in most cases because of the labor involved Yet the data demonstrated that antibiotic activity was present, that the sensitivity of five test species was in approximately the same order as in the agar plate test, and that antibiotic titer was consistently greater in the maltose media The results of a later experiment using medium 8 are given in table 3 Here the antibiotic titer was considerably higher, permitting calculation of the dilution for the 50 per cent inhibition end point Since *Shigella paradysenteriae* (Sonne) proved to be consistently the most sensitive of these five test species, it was decided to employ this culture as the assay organism

*Antiviral and antirickettsial activity* When a Seitz filtrate of an early low potency shaken flask culture in Waksman's streptomycin medium was tested by methods described below, no significant effect was observed against the viruses of St. Louis encephalitis or of type A influenza in mice, but indications of activity against *Rickettsia prowazekii* in chick embryos were obtained These results are presented below in the section on chemotherapy of experimental typhus (figure 2)

#### PRODUCTIVITY OF SUBMERGED CULTURE MEDIA

*Equipment and methods* Chloromycetin has been produced in aerated, submerged culture in several types of containers Erlenmeyer flasks on rotary

<sup>2</sup> Early lots were prepared by Quentin R. Bartz, later lots, by Clark E. Cottrell

shaking machines and laboratory fermenters were employed for exploratory media studies, for the furnishing of material for the development of extraction

TABLE 2

*Turbidimetric data on culture filtrates of the Streptomyces in shaken flasks*

TEST SPECIES	DILU TION	SHAKEN CULTURE MEDIA							
		1	2	3	4	5	6	7	8
		percentage of inhibition							
<i>Escherichia coli</i>	1 10	0	85	89	100	0	73	0	81
	20	—	71	72	100	—	42	—	60
<i>Klebsiella pneumoniae</i> type A	1 10	0	100	93	—	0	90	12	87
	20	—	88	83	100	—	70	14	81
	25	—	—	—	—	—	43	—	—
	40	—	—	55	—	—	—	—	42
	45	—	50	—	—	—	—	—	—
	125	—	—	—	51	—	—	—	—
<i>Salmonella schottmuelleri</i>	1 10	0	87	90	—	0	73	0	84
	20	—	77	77	100	—	50	—	63
<i>Shigella paradysenteriae</i> (Sonne)	1 10	0	92	100	—	0	89	0	94
	20	—	86	85	100	—	73	—	81
<i>Staphylococcus aureus</i>	1 10	0	100	71	90	0	58	0	63
	20	—	85	46	80	—	28	—	32
<i>Streptococcus viridans</i>									
“ PD 04171	1 100	—	—	—	41*	—	—	—	—
“ PD 04150	1 100	—	—	—	55*	—	—	—	—
“ PD 04365	1 100	—	—	—	41*	—	—	—	—
“ PD 04409	1 100	—	—	—	50*	—	—	—	—

\* These values are based upon a filtrate that caused 50 per cent inhibition of *Klebsiella pneumoniae* when diluted 1 200

TABLE 3

*Turbidimetric data on a shaken culture filtrate of the Streptomyces using medium 8*

TEST SPECIES	DILUTIONS					CALC DILUTION FOR 50% INHIB
	1 100	1 200	1 250	1 300	1 400	
	percentage of inhibition					
<i>Escherichia coli</i>	84	60	49	36	21	1 250
<i>Klebsiella pneumoniae</i> A	87	69	54	44	29	270
<i>Salmonella scholtmuelleri</i>	86	62	56	47	36	285
<i>Shigella paradysenteriae</i> (Sonne)	100	84	78	67	57	>400
<i>Staphylococcus aureus</i>	43	17	15	<10	<10	<100

and purification procedures, and for initial chemical work on the properties and structure of crystalline chloromycetin. Larger amounts of culture liquid to

furnish material for further chemical work and for pharmacological studies were produced in horizontal and vertical fermenters. Either spore suspensions from agar culture or vegetative liquid cultures were used as inoculum. The temperature in all equipment was maintained between 23 and 27 C.

*Culture media* Studies on media composition have been made largely on the shaking machines.

Reference has been made above to the superiority of maltose to glucose in various media. Table 4, portraying a typical experiment, shows that, whereas

TABLE 4

*Effect of carbohydrates on biosynthesis of chloromycetin in shaken flasks*

BASE MEDIUM CASAMINO ACIDS 0.5%, B Y FERMENTATION SOLUBLES 0.5%, SODIUM CHLORIDE 0.5%, PLUS		MEAN POTENCIES OF QUADRUPPLICATE FLASKS AFTER 5 DAYS
<i>carbohydrate</i>	<i>per cent</i>	<i>µg/ml</i>
Glucose	1.0	<19
Lactose	1.0	<19
Maltose	1.0	24
Glycerol	1.0	47

TABLE 5

*Effect of various proteins and protein hydrolyzates on biosynthesis of chloromycetin in shaken flasks*

BASE MEDIUM GLYCEROL 1.0%, B Y FERMENTATION SOLUBLES (CSC) 0.5%, SODIUM CHLORIDE 0.5%, PLUS		MEAN POTENCIES OF QUADRUPPLICATE FLASKS AFTER			
<i>protein</i>	<i>%</i>	3 days	4 days	5 days	6 days
		<i>µg/ml</i>			
Soya					
Soybean oil meal (Staley)	0.5	59	60	55	66
Alpha protein (Glidden)	0.5	69	72	71	81
Protein hydrolyzate (Publicker)	0.5	76	89	84	88
Milk					
Labco casein (Borden)	0.5	54	83	92	87
N-Z amine B (Sheffield)	0.5	23	78	82	84
Meat					
Hog stomach residue (P, D & Co.)	0.5	96	118	105	116
Tryptone (Difco)	0.5	136	169	155	164
Peptone (Difco)	0.5	103	106	115	110

results with lactose were of the same order as those with glucose, glycerol was even more effective than maltose in effecting biosynthesis of chloromycetin.

A variety of animal and vegetable proteins and protein hydrolyzates, including milk, corn, soya, wheat, cotton seed, and meat products, were tested for their effectiveness in glycerol media. The meat products were found superior, in most cases, to the others. Difco peptone, Difco tryptone, and a hog stomach residue<sup>3</sup> produced the best yields. The latter is a waste product and was most

<sup>3</sup> A concentrated and dried residue from ground hog stomachs extracted with saline for the production of "ventriculin" (Parke, Davis and Company).

often used because of its availability Table 5 depicts an experiment in which various soya, milk, and meat proteins and hydrolyzates were compared It will be noted that not only were the activities produced by the meat products considerably higher, but these high activities were reached in a shorter period of time than the highest activities produced by the soya or milk products

The replacement of B-Y fermentation solubles (CSC) by molasses has recently been found possible Yeast products, beef extract, distillers solubles, and corn steep solids resulted in lowered activity A comparison of several of these materials is made in table 6 The quantities used had been found in preliminary experiments to be the optimum concentrations in the base medium

TABLE 6  
*Effect of various supplementary materials on biosynthesis of chloromycetin  
in shaken flasks*

BASE MEDIUM GLYCEROL 1 0% HOG STOMACH RESIDUE 0 5% SODIUM CHLORIDE 0.5% PLUS		MEAN POTENCIES OF QUADRUPLICATE FLASKS AFTER			
		3 days	4 days	5 days	6 days
<i>supplementary material</i>	%	<i>µg/ml</i>			
Corn steep solids (Corn Prod Ref)	0 8	25	98	88	83
Brewers' yeast type 2019 (Standard Brands)	0 7	<25	35	33	34
Distillers' solubles (Brown and Forman)	1 0	<25	<25	<25	30
B-Y fermentation solubles (CSC)	0 5	114	119	120	113
Molasses (Brer Rabbit green label)	1 0	142	137	118	137

TABLE 7  
*Course of chloromycetin potency and pH of culture liquid*

AGE	POTENCY	REACTION
<i>hours</i>	<i>µg/ml</i>	<i>pH</i>
0	—	6 70
65	68	6 10
89	82	5 25
97	78	5 79
113	78	5 89

It would appear that there are as yet unidentified substances in both molasses and B-Y fermentation solubles, which is the residue from a molasses fermentation process for the production of industrial alcohol, that are capable of stimulating chloromycetin production by the organism

During the course of the elaboration of chloromycetin by the organism, the pH of the culture liquid tends to drop during the early part of the incubation period It has frequently been observed that the concentration of chloromycetin in the culture liquid is greatest when the pH has fallen to its lowest point or shortly after it has begun to rise again Table 7 shows the course of the pH and the increase in amount of chloromycetin in a typical experiment

## ANTIBIOTIC ACTIVITY OF CRYSTALLINE CHLOROMYCETIN

*In Vitro Activity*

The results of *in vitro* tests of antibiotic activity of crystalline chloromycetin, already reported in part (Ehrlich *et al*, 1947), are summarized in table 8

*Antibacterial activity* Although methods of testing and recording bacteriostatic activity necessarily varied for different species, the results in the first section of table 8 show that chloromycetin is considerably more active in broth culture against several gram-negative species than against the gram-positive and acid-fast species tested. When compared with streptomycin (table 8, notes p and q), chloromycetin is thus approximately one-tenth as active against streptomycin-sensitive strains of *Mycobacterium tuberculosis*, 1 to 2 times as active against *Bacillus mycoides* and *Staphylococcus aureus*, and 2 to 16 times as active against the gram-negative organisms compared. When compared with penicillin (table 8, notes o and p), chloromycetin is 7 to 36 times as active against the gram-negative species tested, approximately one-fiftieth as active against the 209 strain of *Staphylococcus aureus*, and remarkably active against a Schuhardt tick strain of *Borrelia recurrentis* in a 2-hour test in which 100 I U per ml of penicillin are inactive.

The data on *M. tuberculosis* show that chloromycetin maintains its activity sufficiently at 37 C in the presence of beef plasma to prevent growth. They show also that a substrain of *M. tuberculosis* highly resistant to streptomycin is not more resistant to chloromycetin than the streptomycin-sensitive parent strain.

*Antifungal activity* Neither 200 µg of chloromycetin per ml of broth nor 12,500 µg per ml of agar—highest concentrations tested—inhibited the growth of the pathogenic yeasts and filamentous fungi tested.

*Antiprotozoal activity* When *Pelomyxa carolinensis*, a large multinucleate rhizopod, and *Tetrahymena geleni*, a small ciliated protozoan, were placed into chloromycetin-saturated buffered culture solution and into chloromycetin saturated 2 per cent proteose-peptone solution, respectively, the appearance and observable activities of these two free-living species remained unchanged over a 48-hour period. Similarly, when *Trichomonas foetus* was placed in a nearly saturated solution of chloromycetin in 0.7 per cent saline, the organisms were not killed in 7 hours, as compared with 1.5 hours' death time in an equal concentration of calcium penicillin solution. Likewise when *Endamoeba histolytica* was placed in diphasic egg-Locke medium containing graded dilutions of a boiled Locke overlay solution of chloromycetin, the numbers of motile amebae after incubation at 37 C for 48 hours decreased only to an extent attributable to inhibition of the associated mixed bacterial flora.

*Chemotherapy*

A number of tests on experimentally infected animals have been initiated. Certain of these tests are being extended, but the following descriptions are indicative of the activity of chloromycetin in infected animals.

TABLE 8  
Antibiotic activity of chloromycetin *in vitro*

TEST SPECIES AND STRAIN	CULTURE MEDIUM	METHOD OF TESTING	END POINT OBSERVED	CONCENTRATION OF CHLOROMYCETIN REQUIRED  µg/ml
<b>Bacteria</b>				
<i>Bacillus my- coides</i> (PD 04595)	Difco brain heart infusion (pH 7.4)	Broth dilution	50% of growth (turbidimetri- cally) of control after 4-5 hours at 37 C	0.50 <sup>a</sup>
<i>Borrelia recur- rentis</i> <sup>a</sup> (Schu- hardt)	2.5% Rat serum + buffer (pH 7.0)	Buffer dilution	Death within 2 hours at 37 C	2.50 <sup>a</sup>
<i>Borrelia recur- rentis</i> <sup>a</sup> (Schu- hardt)	2.5% Rat serum + buffer (pH 7.0)	" "	50% motile after 2 hours at 37 C	0.00625
<i>Brucella abortus</i> (Huddleson 1335)	Difco tryptose (pH 6.9)	Agar dilution	No growth (by un- aided eye) after 24 hours at 37 C	2.00
<i>Brucella meliten- sis</i> <sup>b</sup> (recent re-isolate from guinea pig)	Difco tryptose (fortified) <sup>c</sup>	Broth dilution	No growth (by un- aided eye) after several days at 37 C	0.50 <sup>d</sup>
<i>Brucella suis</i> <sup>b</sup> (moderate guinea pig virulence)	Difco tryptose (fortified) <sup>c</sup>	" "	No growth (by un- aided eye) after several days at 37 C	0.50 <sup>d</sup>
<i>Eberthella ty- phosa</i> (N I H "Hopkins")	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	0.25 <sup>a</sup>
<i>Escherichia coli</i> (PD 01495)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	0.33 <sup>a</sup>
<i>Hemophilus pertussis</i> , phase I, <sup>e</sup> high mouse virulence (PD 04692)	Sauer's (rabbit) blood	Agar dilution	No growth (by un- aided eye) after 72 hours at 35 C	0.2
<i>Klebsiella pneu- moniae</i> , type A (PD 04544)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	0.33 <sup>a</sup>
<i>Mycobacterium tuberculosis v hominis</i> <sup>o</sup> (H37Rv)	Youmans' syn- thetic <sup>o</sup> (pH 7.0)	" "	No growth (by un- aided eye) after 2 weeks at 37 C	12.5 <sup>a</sup>
<i>Mycobacterium tuberculosis v hominis</i> <sup>o</sup> (H37RvR) <sup>f</sup>	Youmans' syn- thetic <sup>o</sup> (pH 7.0)	" "	No growth (by un- aided eye) after 2 weeks at 37 C	12.5
<i>Mycobacterium tuberculosis v hominis</i> <sup>o</sup> (H37Rv)	Youmans' syn- thetic <sup>o</sup> + 10% beef plasma	" "	No growth (by un- aided eye) after 2 weeks at 37 C	12.5
<i>Mycobacterium tuberculosis v hominis</i> <sup>o</sup> (H37RvR) <sup>f</sup>	Youmans' syn- thetic <sup>o</sup> + 10% beef plasma	" "	No growth (by un- aided eye) after 2 weeks at 37 C	12.5
<i>Pasteurella tularensis</i> <sup>h</sup> (Schu, and Church)	Synder <i>et al</i> , peptone <sup>i</sup>	" "	No growth (turbidimetrically and by plate counts) after 96 hours	0.4-10 <sup>d</sup>

TABLE 8—Continued

TEST SPECIES AND STRAIN	CULTURE MEDIUM	METHOD OF TESTING	END POINT OBSERVED	CONCENTRATION OF CHLORAMPHENICOL REQUIRED
Bacteria— Continued				μg./ml.
<i>Proteus vulgaris</i> (PD 04736)	Difco brain heart infusion (pH 7.4)	Agar dilution	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	0.33
<i>Salmonella</i> <i>schottmuelleri</i> (PD 01180)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	0.33 <sup>a</sup>
<i>Shigella para-</i> <i>dysenteriae</i> (Sonne) (PD 04628)	Difco brain heart infusion (pH 7.4)	Broth dilution	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	0.20 <sup>a</sup>
<i>Staphylococcus</i> <i>aureus</i> (N I H 209)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetri- cally) of control after 3-4 hours at 37 C	1.00 <sup>a</sup>
<i>Streptococcus</i> <i>pyogenes</i> (PD 04472)	Difco brain heart infusion (pH 7.4)	" "	50% of growth (turbidimetri- cally) of control after 5-6 hours at 37 C	0.63
Yeasts and fila- mentous fungi <sup>1</sup>			Results obtained with highest concentration tested	mg./ml.
<i>Candida albi-</i> <i>cans</i> (PD 04600)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	Broth dilution	No inhibition after 24 hours at 37 C	0.20
<i>Candida albi-</i> <i>cans</i> (PD 04600)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	Agar diffusion	No inhibition after 24 hours at 37 C	12.5
<i>Cryptococcus</i> <i>neoformans</i> (N I H 3713)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	Broth dilution	No inhibition after 24 hours at 37 C	0.20
<i>Cryptococcus</i> <i>neoformans</i> (N I H 3713)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	Agar diffusion	No inhibition after 24 hours at 37 C	12.5
<i>Microsporium</i> <i>audouinii</i> (N I H 239)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	Broth dilution	No inhibition after 10 days at 28 C	0.20
<i>Microsporium</i> <i>canis</i> (N I H 237)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	" "	No inhibition after 10 days at 28 C	0.20
<i>Trichophyton</i> <i>interdigitale</i> (N I H 640)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	" "	No inhibition after 10 days at 28 C	0.20
<i>Trichophyton</i> <i>interdigitale</i> (N I H 640)	Sabouraud's glu- cose + neopep- tone (pH 5.8)	Agar diffusion	No inhibition after 6 days at room temperature	12.5
Protozoa			Results obtained with highest concentration tested	mg./ml.
<i>Endamoeba his-</i> <i>tolytica</i> <sup>a</sup> with mixed bac- terial flora (U of Chicago)	Egg—Locke di- phasic (pH 7.9)	Broth dilution in Locke overlay	No significant de- crease in number of motile ameb- ae after 48 hours at 37 C	1.0
<i>Pelomyxa carol-</i> <i>inensis</i> <sup>1</sup>	Pace and Kimura buffer <sup>a</sup> (pH 6.8-7.0)	Sat. soln. in buffer	No change in ap- pearance, loco- motion, etc., during 45 hours at 25 C	2.5



TABLE 8—Concluded

TEST SPECIES AND STRAIN	CULTURE MEDIUM	METHOD OF TESTING	END POINT OBSERVED	CONCENTRATION OF CHLOROMYCETIN REQUIRED
Protozoa— Continued			Results obtained with highest concentration tested	
<i>Tetrahymena geleni</i> <sup>1</sup>	2 0% proteose peptone, Difco (pH 6.8)	Sat soln in broth	No change in ap- pearance, loco- motion, etc., during 48 hours at 25 C	<sup>mg/ml</sup> 2.5
<i>Trichomonas foetus</i> <sup>a</sup> (B. B. Morgan)	0.7% sodium chloride	Saline dilution	No deaths during 7 hours at 37 C	2.0

<sup>a</sup> Tested under the direction of Paul E. Thompson by Donald L. Bush at the Research Laboratories of Parke, Davis and Company, Detroit

<sup>b</sup> Tested by E. H. Kelly and A. N. Gorelick at Camp Detrick, Frederick, Maryland

<sup>c</sup> Fortified with 1 per cent glucose, 0.01 mg per cent thiamine hydrochloride, and 1 mg per cent ferrous sulfate. Organisms were added in 5 per cent volume of a solution containing 0.1 per cent tryptose and 0.5 per cent sodium chloride

<sup>d</sup> These values are tentative and may be modified as the result of repetition

<sup>e</sup> Tested by Guy P. Youmans at the Department of Bacteriology, Northwestern University Medical School, Chicago

<sup>f</sup> Streptomycin-resistant substrain of H37Rv (Williston and Youmans, 1947)

<sup>g</sup> Youmans and Karlson, 1947

<sup>h</sup> Tested by H. T. Eigelsbach and I. W. Gibby at Camp Detrick, Frederick, Maryland

<sup>i</sup> Two per cent Difco peptone, 1 per cent sodium chloride, and 0.1 per cent glucose, adjusted to pH 7.0 before sterilization (Snyder *et al.*, 1946)

<sup>j</sup> Tested under the direction of Arthur B. Hillegas by Bessie D. Moore at the Research Laboratories of Parke, Davis and Company, Detroit

<sup>k</sup> Tested under the direction of Paul E. Thompson by Betty Lou Lilligren at the Research Laboratories of Parke, Davis and Company, Detroit

<sup>l</sup> Tested under the direction of Donald M. Pace by David Russell at the Department of Physiology, University of Nebraska, Lincoln

<sup>m</sup> Pace and Kimura, 1946

<sup>n</sup> Tested by Thomas F. Reutner at the Research Laboratories of Parke, Davis and Company, Detroit

<sup>o</sup> Although penicillin was effective against *Borrelia recurrentis* after prolonged contact, 100 I.U. per ml had no effect in 2 hours (Schuhardt)

<sup>p</sup> Concentrations required for comparable effect

ORGANISM	PENICILLIN		CHLOROMYCETIN	RATIO
	I.U./ml = $\mu\text{g/ml}$		$\mu\text{g/ml}$	P/C
<i>E. coli</i>	14	8.4	0.33	25
<i>K. pneumoniae</i>	4	2.4	0.33	7
<i>S. schottmuelleri</i>	4	2.4	0.33	7
<i>S. paradysenteriae</i> (Sonne)	12	7.2	0.20	36
<i>S. aureus</i>	0.03	0.018	1.00	0.018

ORGANISM	IN DIFCO PENASSAY BROTH		
	STREPTOMYCIN BASE	CHLOROMYCETIN	RATIO
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	S/C
<i>B. mycoides</i>	0.66	0.63	1.05
<i>E. typhosa</i>	1.65	0.36	4.61
<i>E. coli</i>	2.50	0.50	5.00
<i>K. pneumoniae</i>	0.50	0.25	2.20
<i>P. vulgaris</i>	8.00	0.40	16.0
<i>S. schottmuelleri</i>	1.65	0.50	3.32
<i>S. paradysenteriae</i> (Sonne)	1.00	0.33	3.00
<i>S. aureus</i>	1.65	1.00	1.65

<sup>q</sup> Most virulent strains of the tubercle bacillus are completely inhibited under comparable conditions by less than 2.0  $\mu\text{g}$  streptomycin base per ml (Youmans and Karlson, 1947)

<sup>r</sup> Tested by R. W. Sarber at the Research Laboratories of the Parke, Davis and Company, Detroit

*Experimental avian malaria*<sup>4</sup> Starting 6 hours before inoculation with the 12A strain of *Plasmodium lophurae* by the intravenous injection of parasitized blood, groups of three 100-g ducklings were treated twice daily by the intraperitoneal route with chloromycetin in 50 per cent propylene glycol until the untreated controls reached their parasitemia peaks on the fifth day. Maximum tolerated doses (200 mg per kg per day) were without antimalarial effect, as determined by comparison of the parasitemia in the treated birds with that in the untreated controls.

*Experimental rabbit syphilis* Rabbits infected with the Nichols strain of *Treponema pallidum* and receiving chloromycetin intramuscularly at the rate of 25 mg per kg per day in two divided doses for 8 days showed no change in lesions or disappearance of spirochetes. Although daily dosages of 50 and 100 mg per kg cleared the lesions of spirochetes, the effect proved to be temporary.

*Experimental mouse septicemias* Exploratory experiments, using small numbers of mice infected intraperitoneally with lethal doses of virulent *Klebsiella pneumoniae* (type A), *Shigella paradysenteriae* (Flexner), *Shigella paradysenteriae* (Sonne), *Diplococcus pneumoniae* (type I), *Streptococcus hemolyticus*, and *Streptococcus viridans* and treated subcutaneously with chloromycetin in 20 per cent propylene glycol, streptomycin sulfate, or penicillin G, showed that chloromycetin was qualitatively similar to streptomycin but quantitatively inferior in protective action.

*Experimental tuberculosis* *Mouse chemotherapy* Subcutaneous and oral chemotherapeutic tests have been undertaken by Dr. Youmans,<sup>5</sup> who will report in a separate publication.

*Tuberculostatic blood levels* *Guinea pigs*<sup>6</sup> In order to ascertain by a short method whether or not tuberculostatic blood levels of chloromycetin are readily attainable, Drs. Feldman and Karlson performed an "in vivo, in vitro" test with 700-g guinea pigs. Each of two animals received subcutaneously 15 ml of an 0.85 per cent sodium chloride solution containing 2.5 mg chloromycetin per ml every 2 hours for 3 injections, so that each animal received a total of approximately 160 mg chloromycetin per kg of body weight; they were bled 1 hour after the last injection. Each of two animals received by intubation 10 ml of a suspension containing 13.5 mg chloromycetin per ml hourly for 5 doses or a total of 964 mg per kg each; these animals were bled 1 hour after the last injection. The specimens of blood from the treated animals and two specimens from normal guinea pigs were placed in a refrigerator overnight. The serum from each animal was collected from the clot about 16 hours after bleeding and mixed with an equal volume of Proskauer and Beck liquid medium. This mixture was dispensed in 3-ml amounts into test tubes and inoculated with 0.1 mg tubercle bacilli H37Rv. After 10 days of incubation at 37°C it was found that the growth

<sup>4</sup> Tested by Paul E. Thompson, the Research Laboratories of Parke, Davis and Company, Detroit 32, Michigan.

<sup>5</sup> Guy P. Youmans, Northwestern University Medical School, Chicago 11, Illinois.

<sup>6</sup> Determined by William H. Feldman and Alfred G. Karlson, The Mayo Foundation, University of Minnesota, Rochester, Minnesota.

in tubes containing serum from treated animals was grossly equal to that in the control tubes. In view of the previously demonstrated stability of chloromycetin under these *in vitro* conditions, it is concluded that tuberculostatic blood levels were not present at the time when the treated animals were bled.

*Experimental virus infections* Chloromycetin has given negative results against type A influenza virus in eggs and mice and against St. Louis encephalitis virus and fixed rabies virus in mice. Some indication of protection was obtained against Newcastle disease in young chickens, but tests against the causal virus in embryonated eggs were negative.<sup>7</sup> We have done no work with any member of the psittacosis group of viruses, but Smadel and Jackson (1947) have reported positive results.

*Experimental epidemic typhus in chick embryos* Smadel and Jackson (1947) have already reported that chloromycetin has considerable chemotherapeutic activity, under experimental conditions, against several rickettsial agents. Their investigations are continuing and will be reported elsewhere.

The following studies were made early in the development of this drug in connection with the antiviral and antirickettsial screening program in progress in this laboratory.

*Methods* The Breinl strain of *Rickettsia prowazekii*, used throughout the study, has been maintained by yolk-sac passage in 6-day embryonated eggs. The seed for chemotherapeutic tests consists of pools of bacteriologically sterile rickettsia-rich yolk sacs ground as a 10 per cent suspension in buffered milk and stored in sealed ampoules at  $-70^{\circ}\text{C}$ . Prior to use a representative ampoule is thawed and the suspension titrated in 6-day embryonated eggs to determine the dilution required to kill approximately 50 per cent of the embryos upon the administration of 0.5 ml into the yolk sac by the sixth day postinfection.

For the tests, the yolk sacs of groups of 20 to 30 fertile eggs previously incubated for 6 days at  $37.5^{\circ}\text{C}$  are injected with 0.5 ml per egg of the properly diluted seed material through a small hole in the shell over the air space. The hole is sealed with collodion and incubation is continued at  $35^{\circ}\text{C}$  for the remainder of the test. Seventy-two hours after inoculation the eggs are candled, the dead and weak embryos are discarded, and treatment is begun. Unless otherwise stated, all treatment was withheld until after this 72-hour incubation period to allow the infection to become established. Treatment consists of administration of 0.5 ml of the test material into the yolk sac daily for 1 to 6 days. In each experiment, a control group of infected eggs injected with 0.5-ml doses of sterile saline solution according to the same schedule is included. All eggs are candled daily, those containing dead embryos opened and yolk-sac smears made and examined for the presence of rickettsiae by a modification of Macchiavello's technique. Embryos dying before the fourth day postinfection are not considered in the test and those surviving 14 days postinfection are sacrificed and examined for rickettsiae. Those of the latter are considered dead if rickettsiae are demonstrated, and survivors if smears are negative.

<sup>7</sup> Tests conducted by Herman M. Salk of Parke, Davis and Company.

Each group of test embryos is compared with the corresponding control group by calculating the harmonic mean death time<sup>3</sup> for each group and the percentage dying after the fifth day with demonstrable rickettsiae in yolk-sac smears. With this strain of *R. prowazekii* and the dose used it is extremely difficult to demonstrate free rickettsiae in untreated embryos dying before the fifth day and, for this reason, only those embryos dying after the fifth day are included in the calculation of the percentage of positives.

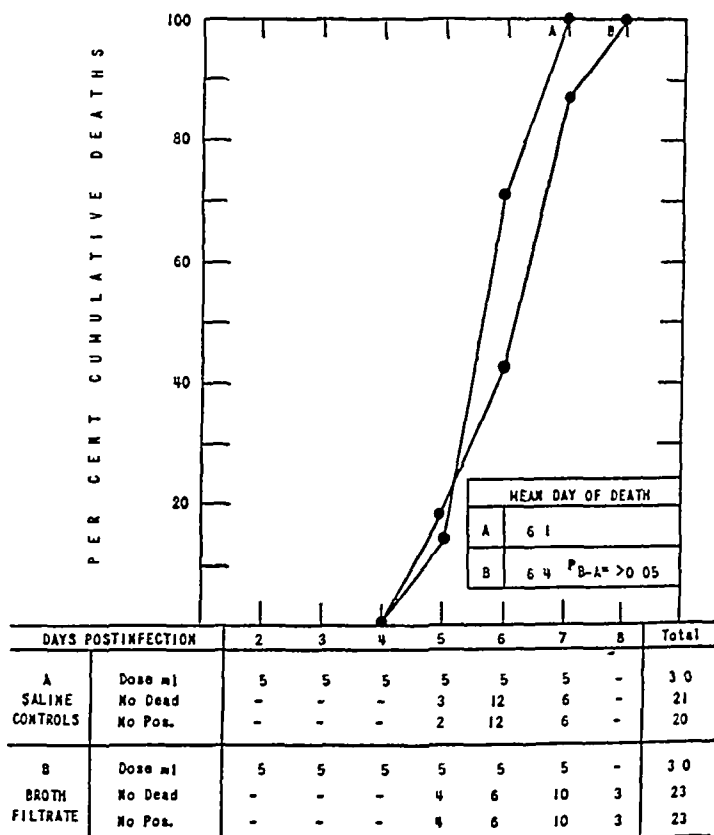


FIG 2 EFFECT OF AN EARLY SHAKEN-CULTURE FILTRATE OF THE STREPTOMYCIN ON *R. PROWAZEKII* INFECTION IN CHICK EMBRYOS

**Experimental results** The first indications of the antirickettsial activity of chloromycetin were obtained from tests on early crude culture filtrates. The results of the first test, with a filtrate, are given in figure 2. Since at that time methods of evaluating activity had not been well developed, the concentration of chloromycetin used is unknown and was undoubtedly very low. A total of 30 ml of the crude broth filtered through a no. 10 Mandler filter candle.

<sup>3</sup> The reciprocal of the mean of the reciprocals or  $1/n(1/x_1 + 1/x_2 + 1/x_3 + \dots + 1/x_n) = 1/\bar{X}$ . This mean is preferable to the arithmetic mean death time since it allows inclusion of survivors inasmuch as  $1/\infty = 0$ .

<sup>4</sup> This work was done under the direction of A. H. Killinger, then of Parke, Davis and Company.

was administered to each egg in 6 daily doses of 0.5 ml each, starting in this case on the second day postinfection. The resulting 0.3-day delay in mean death time is just below the level of statistical significance,<sup>10</sup> however, when considered in conjunction with subsequent work and contrasted with the results on other substances tested during that period, these results probably cannot be attributed to chance alone.

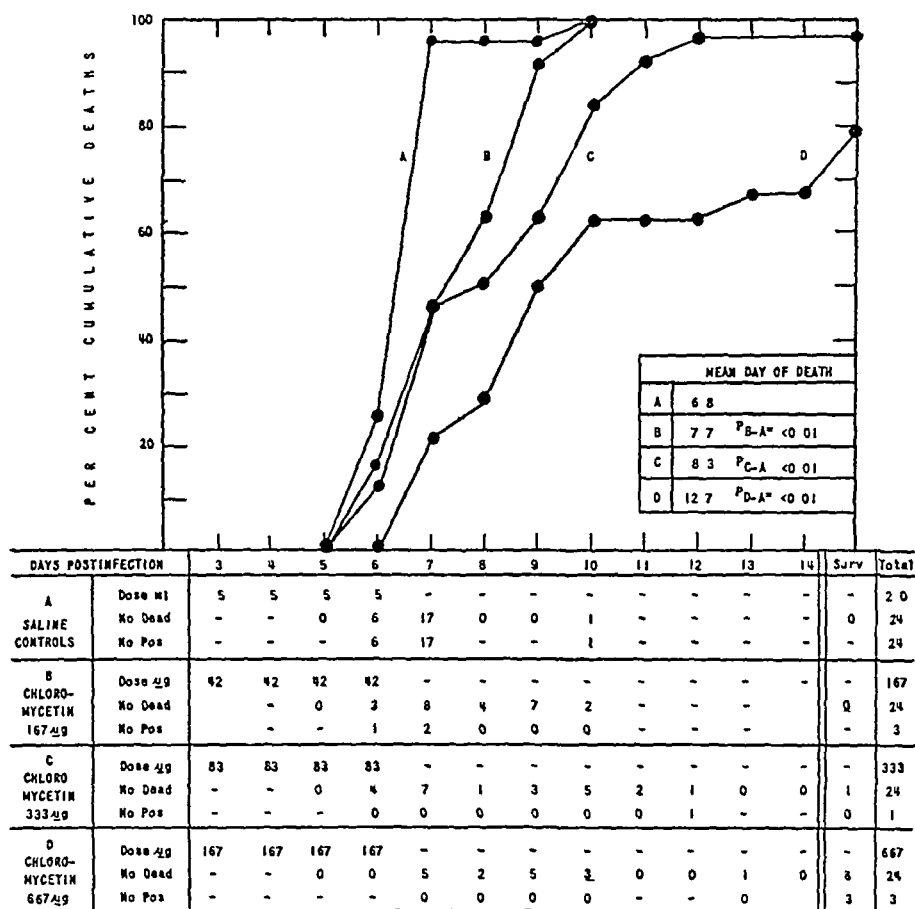


FIG. 3. EFFECT OF INCREASING DOSES OF CHLOROMYCETIN ON EXPERIMENTAL INFECTION OF CHICK EMBRYOS WITH *R. PROWAZEKI*.

When crystalline chloromycetin was obtained, the question of antirickettsial activity was reopened. In the first experiment, treatments were made with a Seitz EK filtrate of an aqueous solution containing 1 mg per 3 ml, and with 1:2 and 1:4 dilutions of this solution in sterile physiological saline. Starting on the third day postinfection, each concentration was administered in 4 daily 0.5-ml doses for totals of 167, 333, and 667 µg per egg, respectively (figure 3, B, C, and

<sup>10</sup> Statistical analysis was made by "Student's" method for measurement data. P in the figures refers to probability that delay in death time could be due to chance alone.

D) Even the smallest dosage resulted in a statistically significant prolongation of mean death time and in marked decrease in demonstrable rickettsiae in the smears. As the total dose was increased, the mean death time was correspondingly prolonged, with the largest dose, 667  $\mu$ g per egg, 8 embryos or 33.5 per cent survived the 14 days' incubation period. When the survivors were sacri-

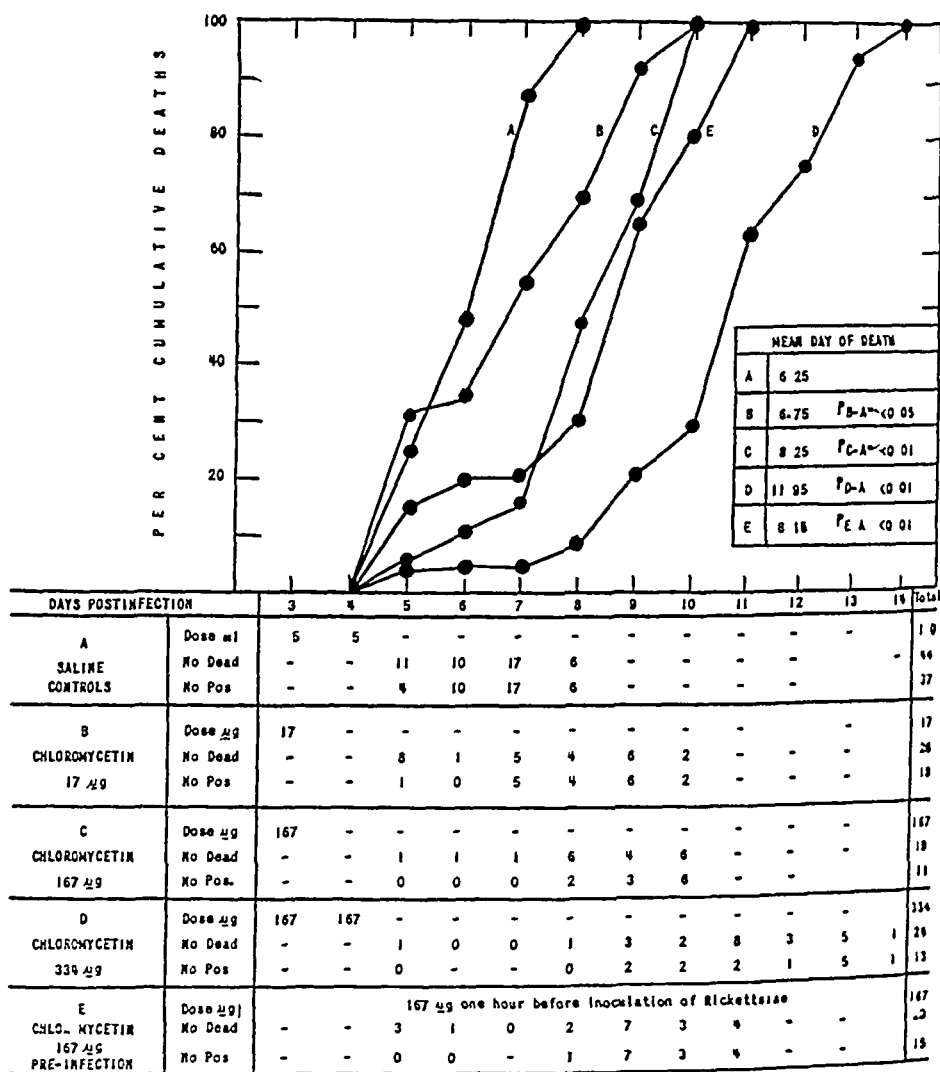


FIG 4. EFFECT OF AMOUNT AND TIME OF STARTING TREATMENT WITH CHLOROMYCETIN ON EXPERIMENTAL INFECTION OF CHICK EMBRYOS WITH *R. PROWAZEKII*

ficed, however, occasional rickettsiae were found in yolk-sac smears from 3 of them. The occurrence of rickettsiae 3 to 5 days after the termination of treatment, associated with a sharp increase in number of deaths, has also taken place in other experiments. Since the drug cannot be excreted from the egg, it must be assumed either that inactivation occurs *in ovo* or that the embryo concentra-

the drug in certain tissues or fluids at the expense of its concentration in the yolk sac

To investigate the possible effect of minimal amounts of chloromycetin and of preinfection and postinfection administration of the drug, the study shown in

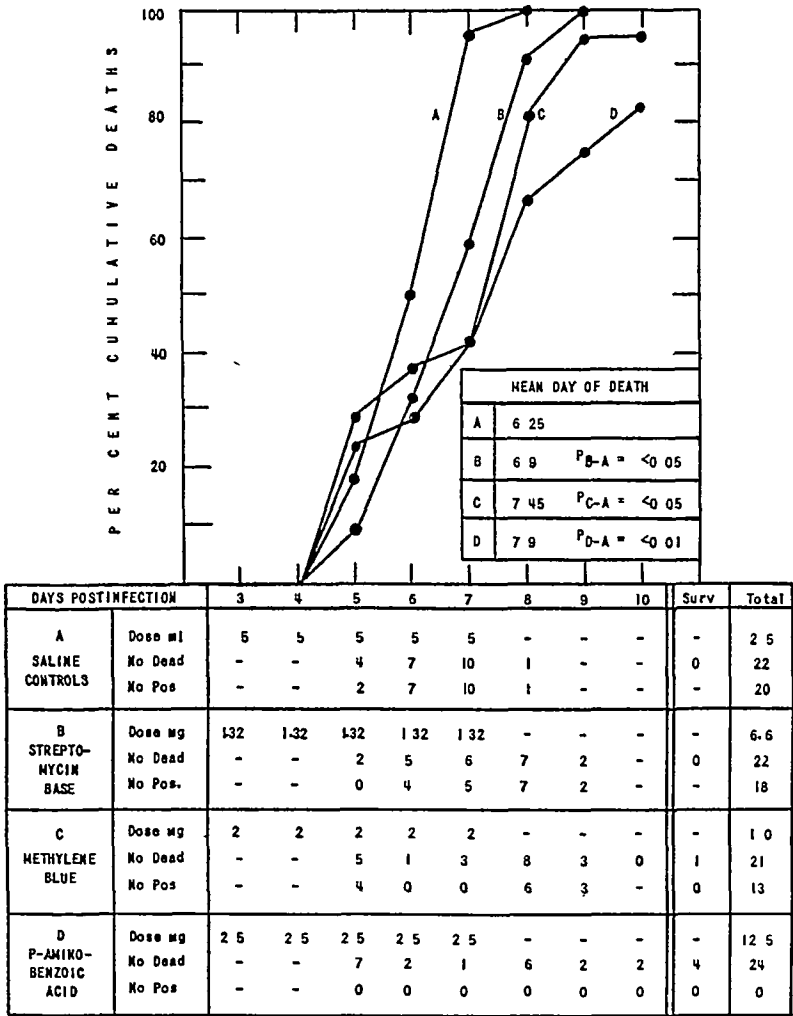


FIG 5 EFFECT OF MASSIVE DOSES OF STREPTOMYCIN, METHYLENE BLUE, AND PARA-AMINO-BENZOIC ACID ON R. PROWAZEKI INFECTION IN THE CHICK EMBRYO

figure 4 was performed It can be seen that as little as 17  $\mu$ g (B) of chloromycetin administered in one dose on the third day postinfection caused a definite prolongation of life and a change in the shape of the cumulative death curve The larger doses, 167  $\mu$ g (C) and 334  $\mu$ g (D), the latter given in two doses, caused increasingly greater prolongations of life Little difference was demonstrated

in the effectiveness of a 167- $\mu$ g dose, whether given 1 hour preinfection (E) or 3 days postinfection (C)

For comparison with chloromycetin the results of treatment with high concentrations of three drugs that have been reported active against experimental infections with rickettsiae (Greiff *et al*, 1944, Hamilton *et al*, 1945, Kikuth and Schilling, 1944, Morgan *et al*, 1947, Smadel, Jackson, and Gauld, 1947) are given in figure 5. This study was made at about the same time and in the same manner as the foregoing experiments and should be comparable in all respects. Relatively large amounts of a low-potency streptomycin hydrochloride, of methylene blue, and of *para*-aminobenzoic acid were given over a 5-day period to three groups of infected embryos (figure 5, B, C, and D). From a comparison of these and similar data, chloromycetin, gram for gram, appears to be decidedly more effective against *R. prowazekii* than any other agent tested under these experimental conditions in chick embryos.

#### PHARMACOLOGY OF CHLOROMYCETIN IN ANIMALS

##### *Toxicity for Small Animals*

When administered intravenously to 20-g white mice in propylene glycol, the maximum tolerated dose of chloromycetin was 200 mg per kg, the LD<sub>50</sub> was approximately 245 mg per kg. Administered orally as an acacia suspension, 1 g per kg produced depression in some animals, with recovery in less than 24 hours, tremors and prostration occurred after 1.25 g per kg, followed by recovery. When 100 mg per kg per day were administered subcutaneously in 20 per cent propylene glycol in two divided daily doses, only slight depression of weight gain was noted in 15 days. Divided subcutaneous doses of 200 mg per kg per day were tolerated at least 11 days, doses above 400 mg per kg per day produced ataxia, weight loss, and death in a few days. Local ulceration occurred at the site of repeated subcutaneous injection. When chloromycetin was administered to 20-g mice for 14 days in a ground diet, normal weight gain occurred at 0.25 per cent concentration (360 mg drug per kg per day), on 1 per cent drug diet (1,290 mg per kg per day) there were no deaths but the mice lost an average of 15 per cent in body weight during the treatment period. Rabbits tolerated 100 mg per kg per day in two daily subcutaneous injections in 20 per cent propylene glycol for at least 8 days.

##### *Dogs*

*Toxicity* Two dogs received acute intravenous doses of chloromycetin as an 8 per cent solution in 75 per cent propylene glycol, injected at the rate of approximately 3.5 ml per minute. The doses employed were 50 and 100 mg per kg. No symptoms were noted other than a transient rise in body temperature (0.5 and 1.1 F) within an hour after injection and a return to normal in several hours.

The effects of intravenous chloromycetin in pure propylene glycol (100 mg or 100 mg per ml of solution) upon blood pressure were tested in four nembutalized



dogs<sup>11</sup> A dose of 12.5 mg per kg injected at the rate of 100 mg of drug per minute was without effect. Single doses of 25, 50, and 100 mg per kg at the same injection rate resulted in declines in blood pressure of 15, 40, and 60 per cent, followed by recovery in 8, 10, and 20 minutes, respectively. A single dose of 150 mg per kg injected at the rate of 450 mg per minute caused sudden death as a result of fall in blood pressure and respiratory failure. One dog survived two 100-mg per kg doses one half-hour apart, but not a third such injection.

On intramuscular injection of a 1.0-g dose of chloromycetin suspended in 3 ml peanut oil into the thighs of three dogs, mild swelling occurred at the injection site in two animals. On autopsy 7 days postinjection, a large thick-walled cyst containing a gelatinous fibrotic mass was found in each injection area. There was little or no cellular infiltration at the periphery of the cyst. When single doses of 150 to 300 mg of chloromycetin in 2 ml of 70 per cent propylene glycol were injected intramuscularly in dogs, considerable pain was evidenced at the time of injection, but no swelling was noted 24 hours later at the injection site, nor was tissue injury evident at autopsy 7 days later.

Four 7- to 14-kg dogs were given chloromycetin twice daily, 5 days a week, for 38 doses during a 24-day experiment. Three of the animals received the antibiotic twice daily as an intramuscular dose of 0.5 g in colloidal solution in 2 ml of 62 per cent propylene glycol (72 to 88 mg per kg per day). One dog received 0.5 g of the drug twice daily in gelatin capsules (143 mg per kg per day).

The three animals receiving chloromycetin by injection gained slightly in body weight (0.25 to 1.0 kg) during the treatment period. A series of injection sites were used in rotation such that each site was reinjected every fourth day. Induration occurred at the sites, and on autopsy the muscle area appeared pale, indurated, and necrotic. Following injections, there was a slight (1 to 2 F) transient rise in body temperature, accompanied by an increase in pulse rate of 15 to 40 beats per minute. Anemia developed in varying degrees in these three animals. In the most severe case, the initial red cell count and hemoglobin percentage ( $5.3 \times 10^6$  per cu mm, 85 per cent) fell to a minimum by the tenth day ( $2.3 \times 10^6$  per cu mm, 42 per cent) and rose to  $3.7 \times 10^6$  per cu mm and 60 per cent, respectively, by the end of the experiment. The anemia in another animal developed gradually during the treatment period, and the final values were approximately those of the first animal. In the mildest case, the initial values ( $5.8 \times 10^6$  per cu mm, 91 per cent) fell to  $4.3 \times 10^6$  per cu mm and 85 per cent by the tenth day and returned to final values of  $5.2 \times 10^6$  per cu mm and 85 per cent.

The one dog receiving chloromycetin by the oral route lost 0.3 kg in body weight during the 24-day treatment period. There were no changes in body temperature or pulse rate associated with drug intake, nor did alteration of the red cell count or hemoglobin value occur.

None of the four dogs receiving parenteral or oral doses showed significant changes in total white cell or differential counts, blood nonprotein nitrogen,

<sup>11</sup> By Graham Chen, the Research Laboratories of Parke, Davis and Company

blood sugar, or bromsulfalein liver function tests, nor were alterations in behavior referable to drug toxicity noted. The urine of all animals remained consistently free of albumin and reducing sugar, and the urinary pH and specific gravity were within normal limits.

**Absorption and excretion** Chloromycetin serum and urine concentrations were determined in each dog on the eighth and twenty-second days of treatment, specimens were taken just prior to the first dose of the particular day (18 hours after the last dose), 2 hours after the first and second doses of the same

TABLE 9  
*Absorption and excretion of single doses of chloromycetin in the dog*

TIME	CONCENTRATION OF CHLOROMYCETIN IN BLOOD AND URINE								
	Intravenous route <sup>a</sup> 19 mg/kg			Intramuscular route <sup>b</sup> 101 mg/kg			Oral route <sup>c</sup> 86 mg/kg		
	Serum	Urine <sup>d</sup>	Urine	Serum	Urine <sup>d</sup>	Urine	Serum	Urine <sup>d</sup>	Urine
hours	μg/ml	μg/ml	μg/specimen	μg/ml	μg/ml	μg/specimen	μg/ml	μg/ml	μg/specimen
0	<6	2-3	—	<1	<1	—	<6	0	—
1/4	17	—	—	—	—	—	—	—	—
1/2	13	—	—	<3	—	—	—	—	—
1	10	—	—	5	—	—	<6	—	—
2	7	644	10,300	7	114	5,590	8	283	1,950
4	<6	426	3,620	5	157	6,440	25	1,360	8,160
6	<6	190	1,900	3	198	5,740	25	2,540	13,950
7	—	—	—	3	184	1,360	—	—	—
8	<6	70	700	—	—	—	20	2,080	16,620
24	<6	9	970	<1	75	16,500	<6	121	17,550
30	—	—	—	—	47	1,360	—	—	—
48	—	—	—	—	8	1,890	—	—	—
Total urinary excretion, μg			17,490				38,880		
Percentage of dose excreted in urine.			8.7				3.9		

<sup>a</sup> A dose of 200 mg drug as a 4 per cent solution in 60 per cent propylene glycol was administered to a 10.5-kg dog.

<sup>b</sup> A 1-gram dose of drug was given suspended in 3 ml peanut oil to a 9.9 kg dog.

<sup>c</sup> A dose of 800 mg in gelatin capsules was given to a 9.3-kg dog.

<sup>d</sup> Urine samples represented the total collection from the time of the previous specimen.

day, and just prior to the first dose of the following day (18 hours after the last dose and 16 hours after the last specimen). There was no indication of slow accumulation of the drug in the body. Intramuscular injections resulted in 2-hour serum levels ranging from less than 1 to 29 μg per ml, the majority of samples (10 out of 12) fell in the range of 2 to 6 μg per ml. The 18-hour levels after injection were usually (11 samples out of 12) in the range of from less than 1 to 2 μg per ml. Urine concentrations of 36 to 406 μg per ml were noted. In

the one orally treated animal, 2-hour serum levels ranged from 6 to 19  $\mu\text{g}$  per ml. Eighteen-hour serum levels were usually less than 2  $\mu\text{g}$  per ml. Urine concentrations were comparable to those obtained on parenteral administration.

Preliminary observations were made on the absorption and excretion of chloromycetin in dogs after a single dose of drug by the oral (1 animal), intravenous (1 animal), and intramuscular (3 animals) routes. Typical data are summarized in table 9. The antibiotic appears to be fairly rapidly excreted or inactivated. The comparable urinary percentage of recoveries of chloromycetin after oral and parenteral administration and the similar drug serum levels during chronic oral and intramuscular administration indicate that a high percentage of the drug is absorbed when given orally.

#### BINDING BY SERUM ALBUMIN

The binding of crystalline chloromycetin by serum albumin was measured<sup>12</sup> by a modification of the method of van Dyke *et al.* (1945) using 3 per cent crystalline bovine serum albumin. Chloromycetin was bound to the extent of 45 per cent, which is intermediate between the values of 38 and 70 per cent found for sulfadiazine and sulfathiazole, respectively, at corresponding equilibrium concentrations. However, in contrast to these agents, the binding of chloromycetin is relatively little affected by variations in drug concentration. Since the degree of adsorption varies markedly with the amount of protein present, it is estimated that the binding by albumin at the blood-stream concentration would approach 60 per cent.

#### DISCUSSION

The work on chloromycetin is still at an early stage, and many pertinent questions await answers. Its antibiotic spectrum *in vitro* is being expanded, and, as indications of activity appear, *in vivo* studies are being undertaken. Since antibacterial *in vitro* studies have already indicated that chloromycetin possesses activity against *Borrelia recurrentis* and several gram-negative pathogens, animal trials with these organisms are proceeding. Of the potentialities of chloromycetin presented in this paper and in that of Smadel and Jackson (1947), the most outstanding is its effectiveness in rickettsial infections of chick embryos and mice. This substance, in small doses, administered either orally or parenterally, afforded greater protection against all the rickettsiae studied than has any other agent yet tested. Furthermore, even the largest doses produced no toxic symptoms in the control animals.

Intravenously in mice and intramuscularly in dogs chloromycetin appears to be well tolerated in single doses of approximately 0.1 g per kg of body weight. Somewhat larger doses are tolerated orally, although further studies are necessary to define the upper limits of tolerance. Anemia develops in dogs on chronic parenteral administration, but it has not yet been noted after oral administration, further studies are in progress to clarify this point. The greater part of a

<sup>1</sup> By John M. Vandenbelt, the Research Laboratories of Parke, Davis and Company.

single dose is excreted or presumably destroyed in 6 to 8 hours. Less than 10 per cent of the dose appears in the urine, indicating extensive inactivation by the body and perhaps excretion by other routes. With repeated doses it appears that serum levels of 2 to 6  $\mu\text{g}$  per ml may be maintained. Binding by serum albumin appears to be sufficiently low to permit satisfactory diffusion into body fluids.

Chloromycetin has not been evaluated in man, and little is known of its action on healthy and diseased animals. Yet the information thus far available suggests certain limitations and advantages that might attend its employment in human medicine. The limited water solubility and the irritant properties of suspensions of the drug on intramuscular injection in dogs appear to contraindicate this route of administration for systemic infections in man, the oral route would appear to be feasible, in view of the apparently high degree of gastrointestinal absorption and favorable tolerance by this route in animals. Should the toxicity of the antibiotic in man prove to be no greater than the data on laboratory animals would indicate, its demonstrated antibiotic activity suggests the possibility of usefulness against certain of the spirochetes, several gram negative species, and notably the rickettsiae.

#### ACKNOWLEDGMENTS

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#### SUMMARY

An unidentified *Streptomyces* has been isolated that produces an antibiotic substance called "chloromycetin." Antibiotic screening tests on solid and in liquid media are described. The organism produces chloromycetin in submerged aerated culture in several types of media, those containing meat proteins, glycerol, and supplementary material such as molasses giving the best yields. A turbidimetric assay method, in which 50 per cent inhibition of growth is the end point, has been developed.

CrySTALLINE chloromycetin has been found *in vitro* to be inactive against yeasts and filamentous fungi, inactive against protozoa, moderately active against gram-positive bacteria and *Mycobacterium tuberculosis*, and active against gram

negative bacteria and *Borrelia recurrentis*. The crystalline antibiotic, *in vivo*, has afforded no protection against avian malaria in ducks, syphilis in rabbits, pneumococcus and streptococcus infections in mice, or against type A influenza, St. Louis encephalitis, and fixed rabies virus infections in mice or in eggs, but moderate protection against *Klebsiella* and *Shigella* infections in mice, and remarkable protection against *Rickettsia prowazekii* in chick embryos.

The toxicity of the antibiotic for laboratory animals is of the order of that of streptomycin. Although chloromycetin is only slightly soluble in water, it may be administered parenterally in propylene glycol solutions, oral administration is also possible, since it appears to be well absorbed from the gastrointestinal tract of the dog. After single doses in the dog, only small amounts of antibacterial substances are excreted in the urine, indicating inactivation in the body and perhaps excretion by other routes.

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## NOTE

### CHANGES INDUCED IN THE O ANTIGENS OF SALMONELLA<sup>1</sup>

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It is possible to induce profound changes in the flagellar antigens of *Salmonella* through growth in media containing H antisera. These changes were recently summarized by Edwards and Moran (Proc Soc Exptl Biol Med, **61**, 242). Hitherto, the only changes reported in the O antigens were those associated with variation between smooth and rough forms and those occurring in natural form variation. In this laboratory attempts to transform O antigens by the method of Boivin *et al* (Experientia, **2**, 139) and by growth in various combinations of S and R antisera and S vaccines have been unsuccessful. However, by a modification of the method of Gard (Z Hyg, **120**, 615) in which absorbed O antisera were used in high concentration, it was possible to bring about certain changes.

When *S. anatum* (III,X,XXVI e,h-l,6) was cultivated in semisolid medium containing III,X,XXVI serum that had been absorbed with a type having O antigens III,XV, the organisms gradually spread through the medium. From the spreading growth was isolated a form that was indistinguishable from *S. newington* (III,XV e,h-l,6) by agglutination and absorption tests. Absorption of the III,X,XXVI serum by *S. newington*, *S. cambridge* (III,XV e,h-l,w), or *S. new-brunswick* (III,XV l,v-l,7) gave the same results. The induced III,XV e,h-l,6 form was then reverted to a typical *S. anatum* strain by cultivation in absorbed III,XV serum. Similarly, *S. meleagridis* (III,X,XXVI e,h-l,w) was changed to a form indistinguishable from *S. cambridge* (III,XV e,h-l,w).

Although filtrates similar to those employed by Boivin were not used in the experiments, it must be remembered that the sera were absorbed with very large doses of bacteria and probably contained dissolved antigens as well as metabolic products. Thus the principle that induced the changes may be the same as that involved in Boivin's work with *Escherichia coli*. Further, attention should be called to the fact that the changes described are only transformations between subgroups of the same O group. Whether similar experiments will permit transformation between distinct O groups or whether they will lead only to rough variation remains to be determined.

<sup>1</sup> The investigation reported in this paper is connected with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U S Public Health Service.





# THE INHIBITION OF SULFHYDRYL ENZYMES AS THE BASIS OF THE BACTERICIDAL ACTION OF CHLORINE<sup>1</sup>

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Columbia University, New York, N Y*

Received for publication November 20, 1947

Current hypotheses of the mode of bactericidal action of chlorine suppose a direct combination of some chemical species of the chlorine with the bacterial protoplasm producing a toxic organic complex (Porter, 1946). Although this view is a great advance over earlier concepts, it still lacks precision in terms of our present knowledge of the intermediary metabolism of cells.

The marked efficiency of chlorine, exerting a bactericidal action in concentrations of 0.2 to 2.0 ppm in water, will at once classify it as a biologically active "trace substance." It may consequently be assumed to exert its effect upon the enzyme systems of the cell (Green, 1941). By investigation of the effect of chlorine on bacteria and on various enzymes, its bactericidal effect has been shown to depend upon the inhibition of certain essential enzyme systems, and the mechanism of this inhibition involves the powerful oxidative action of chlorine on the —SH groups of these enzymes.

## METHODS

Active chlorine was estimated by the acid-iodine thiosulfate titration procedure (Calvert, 1943). Chlorine solutions, when not otherwise specified, were obtained by neutralizing and filtering a fresh solution of calcium hypochlorite.

The various bacteria were grown in Roux bottles on meat infusion agar at 37°C for 16 hours, harvested by centrifuging the saline washings, washed twice with 0.4 per cent saline solution, and stored at 0°C. In later experiments *Escherichia coli* were grown in 8-liter quantities of casein digest broth, aerated by a stream of filtered air, then harvested similarly.

Bacterial suspensions of known nitrogen content were exposed to the chlorine solutions at 37°C for 10 minutes (except in experiments on exposure time, table 2) in the presence of 0.2 M phosphate buffer, pH 7.0. The amount of chlorine used is expressed as  $\mu\text{g}$  of active chlorine per mg of bacterial nitrogen present. In some experiments (tables 1, 2, and 3) any excess chlorine compound remaining after the exposure was removed by the addition of a slight excess of 0.1 N thiosulfate. The suspension was then washed by dilution with water, centrifuged at high speed, and resuspended in the original volume. No differences in metabolic activity or viability were detected between cells treated in this manner and those diluted directly from the chlorine solution for the experiments. The

<sup>1</sup> This investigation was conducted under contract with the Committee on Medical Research of the Office of Scientific Research and Development and at the request of the Office of the Quartermaster General.

metabolic activity of the treated bacterial suspensions and of untreated controls was then determined by the usual Warburg technique. *E. coli*, for example, consistently showed with glucose a  $Q_{O_2}/N$  of 1,000 to 1,200  $\mu$ l.

The viability of the bacteria was determined by subculture of the same suspension used for the manometric determinations. A loopful of the suspension was streaked onto a Difco agar plate and incubated for 12 hours at 37°C, 0 indicated sterility,  $\pm$ , trace of growth, +, sparse growth, and ++, profuse growth. In other experiments with *E. coli*, plate counts were made (figures 1, 2). At the conclusion of the manometric run (15 minutes), a suitable dilution of the bacteria from the cups was made with sterile saline and inoculated into pour plates of Difco Endo agar. Counts recorded are the average of duplicates after 24 hours' incubation.

The aldolase-triosephosphoric dehydrogenase system of muscle was prepared by the method of Green, Needham, and Dewan (1937), the *d*-amino acid oxidase by the method of Warburg and Christian (1938), and the glutamic aspartic transaminase by the method of Green, Leloir, and Nocito (1945). Aldolase from bacteria was prepared by grinding a thick suspension of *E. coli* in a Booth Green mill, centrifuging off the cell debris, dialyzing against dilute  $\text{NaHCO}_3$  overnight, and fractionating with ammonium sulfate. It was assayed by the method of Herbert, Gordon, Subrahmanyam, and Green (1940) with the addition of 0.001 M  $\text{MnSO}_4$  to the reaction.

We wish to thank Dr. Gordon Fair for generous supplies of chlorine and iodine compounds.

#### DETERMINATION OF THE BACTERICIDAL AMOUNT OF CHLORINE

Only those effects of chlorine produced by concentrations equal to, or less than, the bactericidal concentration are germane to the problem of the mechanism of bactericidal action. Preliminary experiments to determine the bactericidal amount under our conditions revealed that the bactericidal effect of chlorine was not simply related to the concentration of chlorine, but depended largely upon the number of bacteria present.

As shown in table 1, more chlorine is required to kill more bacteria. The bactericidal concentration of chlorine therefore varies with the number of bacteria, but the ratio of chlorine to bacteria is remarkably constant. This is shown more precisely in figures 1 and 2. This dependence of bactericidal action upon the amount of chlorine for a given amount of bacteria proved to be experimentally useful, since comparable results could be obtained under widely different conditions. In particular, it provided a value for the bactericidal amount of chlorine which could be applied equally well to bacteria and to enzyme proteins.

The bactericidal range of chlorine amounts was determined more precisely by plate counts of the surviving bacteria. Figure 1 shows the percentage of death in bacterial populations with increasing amounts of chlorine per unit of bacterial N. The inhibition by chlorine of a certain enzyme function, if it is responsible for the bactericidal effect on the bacteria, must occur within a range of chlorine amounts shown in figure 1. Such bactericidal amounts

chlorine are far below the amount causing cell lysis of *E. coli* or nonspecific protein denaturation

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In figure 1 is also given the percentage of inhibition of glucose oxidation with increasing amounts of chlorine per mg of bacterial N, determined in the same experiments as the bactericidal effect. Of the various segments of metabolic

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Active chlorine concentration (μg/ml) Growth (subculture) μg Cl/mg N	CONCENTRATION OF <i>E. COLI</i> (MG N/ML)			
	0.078		0.390	
	13	10	100	66
	0	+	0	+
		130		170

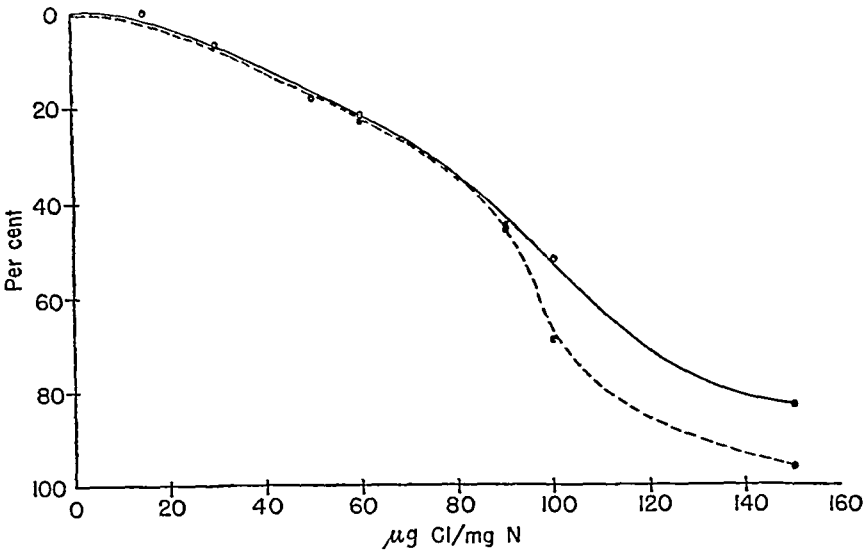


FIG 1 THE PERCENTAGE OF *E. COLI* KILLED (●) AND INHIBITION OF GLUCOSE OXIDATION (○) BY CHLORINE

activity susceptible to measurement, glucose breakdown may be accepted as the reaction most generally essential to all cells. It can be seen that death of the cells parallels the inhibition of glucose oxidation, as would be expected if loss of such essential enzymes caused death. The separation of the curves at higher chlorine concentrations suggests that the relatively resistant cells of the population are those that oxidize glucose more rapidly. This inhibition of glucose oxidation equal to the killing effect has been found consistently in over a hundred experiments under different conditions and occurs with bacteria grown



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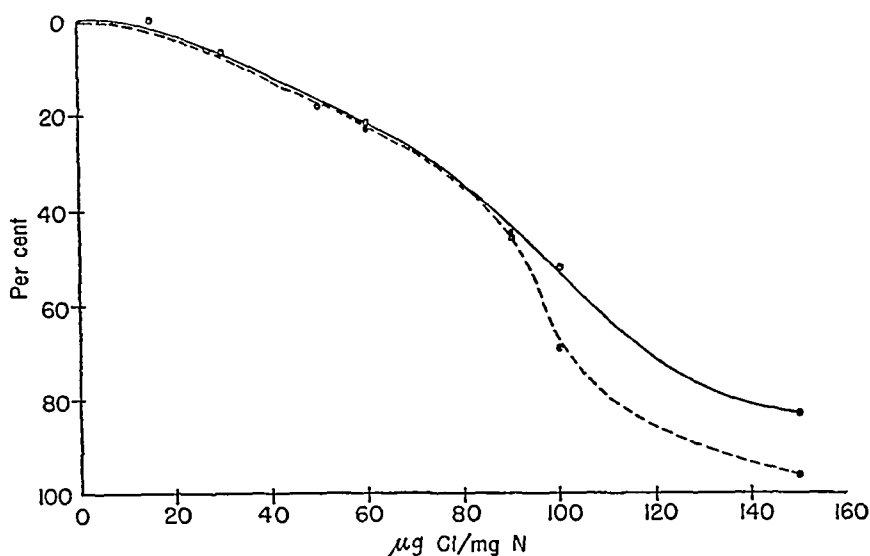


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on different media, aerobically and anaerobically, as well as with a variety of bacterial species

In tables 2 and 3 are given values determined by subculture and manometric experiments showing that sterility of bacterial suspensions occurs only when chlorine has inhibited glucose oxidation fully. In table 2 the effects on glucose oxidation and killing are seen to be parallel for different times of exposure. The effect is complete in 10 minutes under our conditions. In table 3 is shown the

TABLE 2

*Inhibition of glucose oxidation in Escherichia coli suspensions and viability after various times of exposure to chlorine*

Exposure time (minutes)	ACTIVE CHLORINE ( $\mu\text{g}/\text{Cl}/\text{mg N}$ )					
	95			186		
	0 5	5	10	0 5	5	10
Glucose oxidation (per cent inhibition)	16	41	59	95	100	100
Growth (subculture)	++	++	++	+	0	0

TABLE 3

*Effect of various chlorinating agents on oxidation of glucose and viability of bacteria*

	COMPOUND					
	Halazone		Hypochlorite		Succinylchloramide	
<i>Proteus vulgaris</i>						
$\mu\text{g}$ chlorine/mg N	214	106	286	143	214	106
Glucose oxidation (per cent inhibition)	100	91	100	92	100	75
Growth (subculture)	0	±	0	±	0	+
<i>Serratia marcescens</i>						
$\mu\text{g}$ chlorine/mg N	214	106	286	100	160	80
Glucose oxidation (per cent inhibition)	100	50	100	40	100	20
Growth (subculture)	0	++	0	++	0	++
<i>Aerobacter aerogenes</i>						
$\mu\text{g}$ chlorine/mg N	214	106	286	143	160	80
Glucose oxidation (per cent inhibition)	100	79	100	95	100	71
Growth (subculture)	0	++	0	±	0	++
<i>Escherichia coli</i>						
$\mu\text{g}$ chlorine/mg N	237	117	286	188	188	34
Glucose oxidation (per cent inhibition)	100	77	100	83	100	52
Growth (subculture)	0	++	0	+	0	++

parallelism of inhibition of glucose oxidation and bacterial death for various chlorine compounds in several bacterial species

This correlation of glucose oxidation inhibition with cell death is sufficient, general to suggest that chlorine acts by specifically inhibiting one or more of the enzymes of glucose oxidation. Since the integrity of this system is essential for viability, the cell dies. The possibility that the inhibition of glucose oxidation is a result of cell death, rather than a cause, is unlikely. Bacteria killed by



other means, such as acetone or ultrasonics, can still oxidize glucose. Further proof of this view is given in table 4. Iodoacetic acid, known to inhibit glucose breakdown specifically, likewise causes a parallel inhibition of glucose oxidation and cell viability.

#### CHLORINE AS AN —SH ENZYME INHIBITOR

Although chlorine has not previously been shown to act on —SH groups, its effect on several classical sulfhydryl enzymes shows it to be an exceptionally good inhibitor. The inhibition of papain (table 5), for example, is sufficiently

TABLE 4

*Effect of iodoacetic acid on glucose oxidation and growth of Escherichia coli*

(Growth determined after 10 hours' incubation of bacteria plus iodoacetate in 3 ml of meat infusion broth, pH 7. Glucose oxidation measured with bacterial suspension after 2 hours' exposure to iodoacetic acid in phosphate buffer, pH 7)

CONCENTRATION OF IODOACETIC ACID	GROWTH	PER CENT INHIBITION OF GLUCOSE OXIDATION
<i>ppm</i>		
1,200	0	100
600	0	100
120	+	54
60	++	0
0	+++	0

TABLE 5

*Effect of chlorine concentrations of 1 to 10 ppm on inhibition of papain action (0.26 mg enzyme N/ml)*

CONCENTRATION OF CHLORINE		1 CLOTTING TIME	INHIBITION
<i>ppm</i>	<i>μg/mg N</i>	<i>minutes</i>	<i>per cent</i>
0	0	1.5	0
0.7	2.7	1.1	27
1.4	5.4	0.86	43
4.2	16.2	0.43	71
8.4	32.3	0.24	85

great that it has been used for an extremely sensitive assay of free chlorine in water supplies (Green and Stumpf, 1946). In table 6 the effect of different chlorine compounds on the extremely sensitive sulfhydryl system, zymohease-triosephosphate dehydrogenase from rabbit muscle, described by Herbert, Gordon, Subrahmanyam, and Green (1940), and on *D*-amino acid oxidase and transaminase, identified as sulfhydryl enzymes by Singer and Barron (1945), is compared with the inhibition of glucose oxidation in *E. coli*. Similarly, succinic oxidase is inhibited by low amounts of chlorine, but catalase, a nonsulfhydryl enzyme, is not affected.

Chlorine may, therefore, be classed with that group of sulfhydryl enzyme

inhibitors acting by oxidation. It has the property common in this group of producing an irreversible inhibition of the enzyme. All attempts to reverse the chlorine effect on enzymes or bacteria, after it was once established, by the addition of cysteine or glutathione were unsuccessful.

Consideration of the impressive array of —SH enzymes collected by Barron

TABLE 6

*Amount of chlorine for complete inhibition of several animal sulfhydryl enzyme systems compared with glucose oxidation in Escherichia coli*

(500  $\mu\text{g/ml}$  of N in all experiments. Figures refer to active chlorine concentration in  $\mu\text{g/mg N}$ )

REAGENT	GLUCOSE OXIDATION OF E. COLI	ALDOLASE TRIOSEPHOSPHATE DEHYDROGENASE	$\delta$ AMINO ACID OXIDASE	TRANSAMINASE
Hypochlorite	159	27	270	1,360
Halazone	127	22	—	—
Succinylchlorimide	82	22	270	1,090

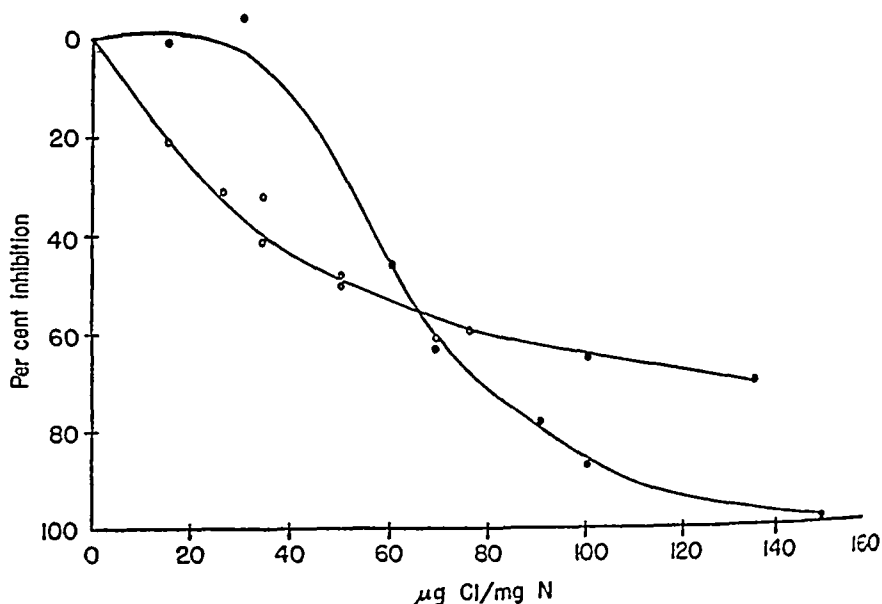


FIG. 2. THE PERCENTAGE OF INHIBITION OF E. COLI GLYCOLYSIS (●) AND OF THE ISOLATED ALDOLASE (○) BY CHLORINE

and Singer (1945) leaves no doubt that such an effective —SH reagent as chlorine could cause cell death by the interruption of essential metabolic systems, probably at several loci, and produce the observed effects on glucose oxidation and viability. It remained only to be demonstrated, therefore, that the inhibition of glucose oxidation by chlorine causing cell death could indeed be referred to a sufficiently sensitive enzyme in the bacteria.

IDENTIFICATION OF ALDOLASE AS A CHLORINE-SENSITIVE  
ENZYME IN *E. COLI*

Although it is quite probable that several enzymes may possess the characteristics sensitizing them to the action of chlorine, and that a multiple effect of chlorine occurs in the cell's metabolism, attempts were made to identify at least one such locus by studying different segments of the glucose-oxidizing system.

Figure 2 shows the inhibition by chlorine of glycolysis in *E. coli*. Although the full relationship of the glycolytic to oxidative cycles for *E. coli* has not been worked out, glycolysis occurs similarly in yeast and muscle (Utter and Werkman, 1941) and may be assumed to represent part of the glucose oxidation pathway. An enzyme of the glycolytic system in muscle, the aldolase-triose-phosphoric dehydrogenase, is shown in table 6 to be easily sensitive enough to account for the bactericidal effect if a similar enzyme exists in the bacteria.

The aldolase enzyme of *E. coli* has been studied by Utter and Werkman (1941). They concluded from their study that the bacterial enzyme was identical with the analogous enzymes of muscle and yeast. This enzyme was prepared by us (see Methods) and found to be sensitive to the various oxidizing sulfhydryl reagents such as chlorine, ferricyanide,  $H_2O_2$ , and iodosobenzoic acid, but not alloxan. It was unaffected by the alkylating or mercaptide-forming sulfhydryl reagents. It differed from other aldolases in its specific requirement for  $Mn^{++}$  for full activity. In figure 2 the chlorine inhibition of this partially purified enzyme prepared from *E. coli* is presented along with the inhibition of glycolysis in the intact cells. It can be seen that the aldolase of *E. coli* is sensitive to chlorine in the same degree as glycolysis and glucose oxidation. The failure to obtain 100 per cent inhibition may be a property of the *in vitro* system, which is tested in the presence of cyanide. However, the effect on aldolase can only be considered representative of the effects on other  $-SH$  enzymes in the cell, and the sum of these effects on the various  $-SH$  enzymes would be expected to approximate the effect on glycolysis shown in figure 2.

## DISCUSSION

In the determination of the mode of action of any germicide or chemotherapeutic agent on biological systems, the essential information is the ultimate action of the compound on a specific metabolic process. To this end we have neglected consideration of the chemical species of the active chlorine and of its permeation into the cell. We may assume that hypochlorous acid is the active form, and that penetration occurs as the un-ionized hypochlorous acid or chloramine (Marks, Wyss, and Strandkov, 1945). Similarly, the possibility that amino groups of various peptides are chlorinated in the cell has not been discussed. The specific effect on metabolism by oxidation of sulfhydryl groups of essential enzymes by the chlorine in itself suffices to explain the observed phenomena. Intracellular chlorination of nitrogen compounds, followed by dissociation of chloramines, would eventually produce the same oxidative result. Such combination when involving extracellular organic material would, of course,

increase the amount of chlorine needed for killing (chlorine demand), but this again is not relevant to the problem of mode of action (Chang and Fair, 1941)

The rationale of these experiments has subsequently been useful in elucidating the mode of action of iodine, organic mercurials, and cationic surface active agents as germicides. It depends upon the determination of the amount of agent necessary to produce the biological effect, in this case the bactericidal effect. This effect must be exerted through some of the enzyme reactions producing the biological phenomenon, and must act on these enzymes in the same amount necessary to produce the effect in the intact cell. Identification of the enzymes sensitive to this amount of agent will show the locus of action, and will frequently give information as to the actual chemical mechanism of the effect on the enzymes by virtue of other known characteristics of these enzymes.

We wish to acknowledge Dr. Harry M. Rose's helpful criticism of this work.

#### SUMMARY

Chlorine in bactericidal amounts or less inhibits various sulfhydryl enzymes and other enzymes sensitive to oxidation. Inhibition of essential enzymes in this way causes death of the bacterial cell, inhibition of glucose oxidation is paralleled by the percentage of bacteria killed. The aldolase of *Escherichia coli* has been shown to be one of the essential enzymes of glucose oxidation sufficiently sensitive to chlorine to explain its bactericidal effect.

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# A VARIANT OF MYCOBACTERIUM RANAE REQUIRING STREPTOMYCIN FOR GROWTH

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Miller and Bohnhoff (1947) in a recent publication have described a meningococcus variant that requires streptomycin for reproduction *in vitro* and *in vivo*. A type b *Hemophilus influenzae* whose growth is favored by a medium containing streptomycin has been reported by Alexander and Leidy (1947). These differ from the usual streptomycin-resistant variants, which grow equally well in the presence or absence of the drug. The present report deals with the isolation of a streptomycin-resistant variant of *Mycobacterium ranae* that grows only in the presence of streptomycin. This variant is also resistant to the sulfonamide drugs and has other unusual characteristics. The variant was isolated in studies dealing with the incidence of spontaneously occurring resistant forms of *M. ranae* in media containing different concentrations of streptomycin.

*M. ranae* was chosen for the preliminary studies because it has been shown (Middlebrook and Yegian, 1946) to develop streptomycin resistance rapidly *in vitro*. It is a fast-growing, acid-fast bacillus that is nonpathogenic for mammals. When large populations of the parent susceptible strain are plated out on a medium containing streptomycin, a small percentage of resistant organisms is consistently obtained. For example, in a population of approximately 400 billion organisms, 20 colonies will grow in the presence of 1  $\mu\text{g}$  per ml of streptomycin. In 10  $\mu\text{g}$  per ml, 4 colonies will grow, and only 1 colony will grow out on a medium containing 100  $\mu\text{g}$  per ml of the drug. Those organisms selected by 100  $\mu\text{g}$  per ml were also resistant to 1,000  $\mu\text{g}$  per ml. The colonies isolated on 1  $\mu\text{g}$  per ml showed a wide range in their degree of resistance. When these were plated out, 5 million organisms yielded 1 colony that would grow in the presence of 100  $\mu\text{g}$  per ml, whereas only 1 colony resistant to 1,000  $\mu\text{g}$  per ml was isolated from a population of 200 million organisms. The resistant organisms (variant A) grow rapidly and luxuriantly on glycerol nutrient agar either in the presence or absence of streptomycin.

The possibility of isolating another variant from this resistant strain that might require streptomycin for growth was considered. The following method proved successful in isolating such a variant.

A deficient medium was prepared which did not support growth of either the parent culture or variant A, and to this was added 1,000  $\mu\text{g}$  per ml of streptomycin<sup>1</sup>. Plates of this medium were inoculated with 0.5 ml (approximately

<sup>1</sup> Composed of the following: asparagine, 0.5 per cent, ammonium citrate, 0.5 per cent, potassium phosphate (dihydrogen), 0.3 per cent, sodium carbonate anhydrous, 0.3 per cent, sodium chloride, 0.2 per cent, magnesium sulfate, 0.1 per cent, ferric ammonium citrate, 0.005 per cent, agar, 1.5 per cent, and streptomycin, 1,000  $\mu\text{g}$  per ml. Final pH 7.2

25 billion organisms) of a suspension of resistant variant A. After 10 to 15 days of incubation each plate showed from 5 to 7 colonies that were 1 to 2 mm in diameter. These colonies (B) were dense and dry, and were unlike those of either the parent or variant A on glycerol agar. There was no increase in pigmentation, and the cellular morphology was not altered, as was shown by the Ziehl-Neelsen technique.

The B colonies were removed with a needle and heavily seeded on fresh deficient medium. After prolonged incubation there was evidence of slight growth but no definite gross colony formation, and repeated subcultures did not show any appreciable increase in growth. It was obvious that this variant B could not be cultured on the deficient medium. Glycerol nutrient agar to which were

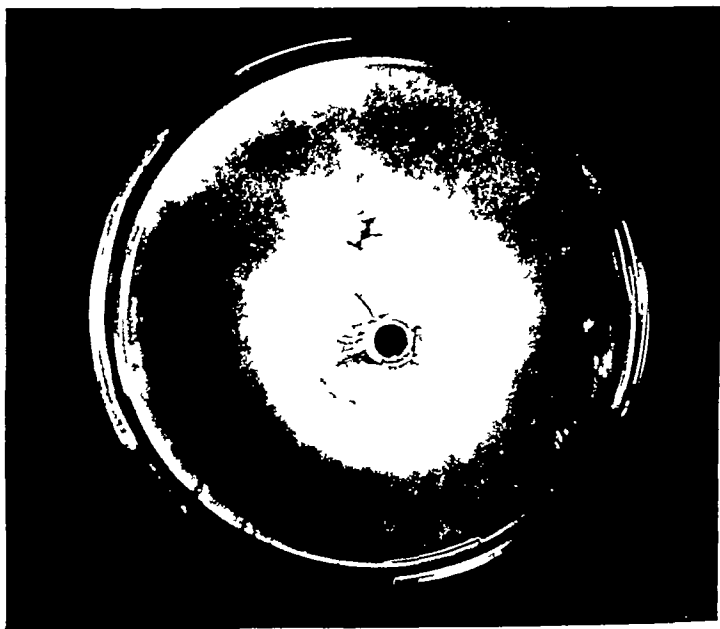


FIG 1 SHOWING GROWTH OF *M. RAVAE* ONLY IN THE AREA WHERE STREPTOMYCIN HAS DIFFUSED INTO THE MEDIUM AT SUFFICIENT CONCENTRATION

added 1,000  $\mu\text{g}$  per ml of streptomycin yielded luxuriant growth after a few days of incubation. In the absence of the drug, the nutrient medium does not support growth of variant B even after prolonged incubation, whereas the parent and variant A show abundant growth within 2 days.

That variant B requires streptomycin for growth can be further demonstrated by the cylinder plate method used in penicillin and streptomycin assay. A drop of bacillary suspension is distributed evenly over the surface of a glycerol nutrient agar plate, and streptomycin is placed in a glass cylinder lightly imbedded in the surface of the medium. After incubation for 4 or 5 days, growth will be seen only in the area in which the drug diffused in sufficient concentration (figure 1).

Other studies with both liquid (Dubos and Davis, 1946) and solid media have shown that, whereas there is visible growth of variant B after 4 days' incubation in the presence of 50  $\mu$ g per ml of streptomycin, the maximum growth is only obtained when the concentration of the drug is 100 or more  $\mu$ g per ml. A trace of growth in 5  $\mu$ g per ml and good growth in 10  $\mu$ g per ml may be secured by prolonging the incubation period 10 days. On subculture from 10  $\mu$ g per ml the organisms grew rapidly in 5  $\mu$ g per ml, and on further transfer good growth was obtained in as low as 1 to 2  $\mu$ g per ml. The results were the same when streptomycin was used from different sources and different batches.

Variant B is also unusual with regard to sulfonamide sensitivity. The parent strain and the streptomycin-resistant variant A are inhibited by 1 mg per cent of sulfathiazole. The variant B will grow in medium containing 100 mg per cent of this drug in the presence of the streptomycin necessary for its growth. Since this culture had not previously been in contact with the sulfonamides, it would seem that in this case resistance was not the result of some action of the drug on the microorganisms.

#### SUMMARY

The isolation of a variant of a streptomycin-resistant nonpathogenic *Mycobacterium* which requires streptomycin for growth *in vitro* has been described. If such a variant can also develop from susceptible parent strains or streptomycin-resistant variants of cultures of pathogenic tubercle bacilli, its significance in the chemotherapy of tuberculosis is evident. Studies along this line are in progress.

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## STUDIES ON SUBTILIN FASTNESS IN VITRO<sup>1,2</sup>

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In previous communications (Salle and Jann, 1945, 1946a,b,c,d, Salle, 1947) subtilin was reported to exert a powerful antibiotic action against a number of gram-positive organisms and certain gram-negative cocci. This agent was bacteriostatic in high dilution and bactericidal in more concentrated solution.

Subtilin exhibited an extremely low toxicity index to living embryonic chick heart tissue fragments cultivated *in vitro*. Under the conditions of the test the antibiotic was shown to be approximately 20 times more toxic to *Staphylococcus aureus* (FDA strain) than to chick heart tissue, a remarkably low tissue toxicity for a chemotherapeutic agent.

Subtilin exhibited a powerful action on the course of experimental pneumococcus type III, *Bacillus anthracis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* infections in animals. The animals responded quickly to treatment without the appearance of any toxic symptoms.

In order that subtilin may be employed to greatest advantage chemotherapeutically it is important to know whether organisms treated with sublethal concentrations develop a resistance or fastness to the antibiotic.

### DEVELOPMENT OF FASTNESS TO SUBTILIN

The general procedure was as follows. Subtilin was added to a series of broth tubes in decreasing concentrations. The final volume in all tubes was 10 ml. To each tube was added 0.1 ml of a 24-hour culture of the test organism. The tubes were incubated at 37°C for 48 hours, then examined for turbidity. The tube showing growth in the greatest concentration of subtilin was used to inoculate a second series, using the same dilutions of the antibiotic. This same procedure was continued through several transfers.

*Development of fastness by Staphylococcus aureus*. A series of broth tubes containing subtilin in dilutions of 1:1,000 to 1:400,000 was prepared. To each tube was added 0.1 ml of a 24-hour nutrient broth culture of *Staphylococcus aureus*, ATC 6538 strain. The tubes were incubated at 37°C for 48 hours, then examined for turbidity. The results are given in table 1. It may be seen that the greatest concentration of subtilin permitting growth was 1:400,000.

Another series of tubes was prepared as described above but inoculated from the tube in the first series containing 1:400,000 subtilin. The tubes were incubated at 37°C for 48 hours, then examined for growth. It may be seen that the greatest concentration of subtilin permitting growth was 1:200,000.

<sup>1</sup> The subtilin preparation used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, California.

<sup>2</sup> This work was aided by a grant from the Antibiotics Study Section, National Institute of Health, U. S. Public Health Service, Washington, D. C.

This procedure was continued through four more series. The results show that *Staphylococcus aureus* developed a gradual resistance or fastness to the antibiotic. In the first series, a dilution of 1:400,000 of subtilin just permitted growth of the organism, whereas in the sixth series a concentration of 1:1,000 was required to produce the same effect.

*Development of fastness by Mycobacterium phlei*. The same procedure was followed as in the preceding experiment except that *Mycobacterium phlei* was substituted for *Staphylococcus aureus*, and transfers were carried through nine series instead of only six. The results are recorded in table 2.

TABLE 1  
*Development of subtilin fastness by Staphylococcus aureus*

SUBTILIN DILUTION	PARENT STRAIN 1ST SERIES	2ND SERIES	3RD SERIES	4TH SERIES	5TH SERIES	6TH SERIES
1:1,000	—	—	—	—	—	+
1:1,111	—	—	—	—	—	+
1:1,250	—	—	—	—	—	+
1:1,428	—	—	—	—	—	+
1:1,666	—	—	—	—	—	+
1:2,000	—	—	—	—	+	+
1:2,500	—	—	—	—	+	+
1:3,333	—	—	—	—	+	+
1:5,000	—	—	—	—	+	+
1:10,000	—	—	—	+	+	+
1:11,111	—	—	—	+	+	+
1:12,500	—	—	—	+	+	+
1:14,285	—	—	—	+	+	+
1:16,666	—	—	—	+	+	+
1:20,000	—	—	—	+	+	+
1:25,000	—	—	—	+	+	+
1:33,333	—	—	+	+	+	+
1:50,000	—	—	+	+	+	+
1:100,000	—	—	+	+	+	+
1:200,000	—	+	+	+	+	+
1:300,000	—	+	+	+	+	+
1:400,000	+	+	+	+	+	+

+ = growth, — = no growth

It may be seen that *Mycobacterium phlei* developed a fastness to the antibiotic. This change, however, occurred at a slower rate than in the case of *Staphylococcus aureus*.

*Development of fastness by Escherichia communio*. In a previous communication (Salle and Jann, 1945) it was stated that the highest dilution of subtilin capable of affecting the growth of certain gram-positive organisms had no effect on the growth of gram-negative bacteria. However, if greatly increased concentrations are employed, gram-negative bacteria become susceptible to the antibiotic. It was found that the gram-negative organism *Escherichia com-*

*munior* readily developed a fastness to subtilin when exposed to sublethal concentrations of the antibiotic. The results are given in table 3.

It may be concluded from these experiments that *Staphylococcus aureus* (gram

TABLE 2  
*Development of subtilin fastness by Mycobacterium phlei*

SUBTILIN DILUTION	PARENT STRAIN 1ST SERIES	2ND SERIES	3RD SERIES	4TH SERIES	5TH SERIES	6TH SERIES	7TH SERIES	8TH SERIES	9TH SERIES
1 1,428	—	—	—	—	—	—	—	—	—
1 1,666	—	—	—	—	—	—	—	—	—
1 2,000	—	—	—	—	—	—	—	—	+
1 2,500	—	—	—	—	—	—	—	—	+
1 3,333	—	—	—	—	—	—	—	+	+
1 5,000	—	—	—	—	—	—	—	+	+
1 10,000	—	—	—	—	—	—	+	+	+
1 11,111	—	—	—	—	—	—	+	+	+
1 12,500	—	—	—	—	—	—	+	+	+
1 14,285	—	—	—	—	—	—	+	+	+
1 16,666	—	—	—	—	—	—	+	+	+
1 20,000	—	—	—	—	—	—	+	+	+
1 25,000	—	—	—	—	—	—	+	+	+
1 33,333	—	—	—	—	—	+	+	+	+
1 50,000	—	—	—	—	—	+	+	+	+
1 100,000	—	—	—	—	+	+	+	+	+
1 200,000	—	—	—	+	+	+	+	+	+
1 300,000	—	—	+	+	+	+	+	+	+
1 400,000	—	+	+	+	+	+	+	+	+
1 500,000	+	+	+	+	+	+	+	+	+

TABLE 3  
*Development of subtilin fastness by Escherichia communior*

SUBTILIN DILUTION	PARENT STRAIN 1ST SERIES	2ND SERIES	3RD SERIES
1 1,000	—	—	+
1 1,111	—	—	+
1 1,250	—	—	+
1 1,428	—	—	+
1 1,666	—	—	+
1 2,000	—	—	+
1 2,500	—	+	+
1 3,333	—	+	+
1 5,000	+	+	+
1 10,000	+	+	+

+, *Mycobacterium phlei* (acid-fast), and *Escherichia communior* (gram —), when exposed to sublethal concentrations of subtilin, readily developed a resistance or fastness to the antibiotic.

## STABILITY OF SUBTILIN-FAST STRAINS

It was considered of great interest and importance to determine for how many generations resistance to subtilin could be retained *in vitro*. Accordingly,

TABLE 4

*Stability of subtilin-fast Staphylococcus aureus ATC 6538 that multiplied in a 1 400,000 concentration of subtilin in broth (table 1)*

CONCENTRATION OF SUBTILIN	PARENT STRAIN	SUBCULTURE						
		1st	5th	12th	18th	23rd	29th	39th
1 33,333	—	—	—	—	—	—	—	—
1 50,000	—	—	—	—	—	+	+	+
1 100,000	—	+	—	—	—	+	+	+
1 200,000	—	+	+	+	+	+	+	+
1 300,000	—	+	+	+	+	+	+	+
1 400,000	+	+	+	+	+	+	+	+

TABLE 5

*Stability of subtilin-fast Staphylococcus aureus ATC 6538 that multiplied in a 1 200,000 concentration of subtilin in broth (table 1)*

CONCENTRATION OF SUBTILIN	SUBCULTURE					
	3rd	5th	11th	24th	30th	91st
1 20,000	—	—	—	—	—	—
1 25,000	—	+	—	—	+	—
1 33,333	—	+	+	+	+	—
1 50,000	+	+	+	+	+	+

TABLE 6

*Stability of subtilin-fast Staphylococcus aureus ATC 6538 that multiplied in a 1 33,333 concentration of subtilin in broth (table 1)*

CONCENTRATION OF SUBTILIN	SUBCULTURE					
	1st	3rd	9th	22nd	28th	86th
1 5,000	—	—	—	—	—	—
1 10,000	+	+	+	+	+	—
1 11,111	+	+	+	+	+	—
1 12,500	+	+	+	+	+	—
1 14,285	+	+	+	+	+	—
1 16,666	+	+	+	+	+	—
1 20,000	+	+	+	+	+	—
1 25,000	+	+	+	+	+	+

subtilin-resistant strains of *Staphylococcus aureus* and *Mycobacterium phlei* were subcultured daily to broth, then tested at certain intervals for their ability to grow in various concentrations of subtilin

TABLE 7

*Stability of subtilin fast Staphylococcus aureus ATC 6538 that multiplied in a 1 10,000 concentration of subtilin in broth (table 1)*

CONCENTRATION OF SUBTILIN	SUBCULTURE				
	1st	7th	13th	25th	86th
1 2,500	—	—	—	—	—
1 3,333	+	+	—	—	—
1 5,000	+	+	+	—	—
1 10,000	+	+	+	+	—
1 11,111	+	+	+	+	—
1 12,500	+	+	+	+	—
1 14,285	+	+	+	+	—
1 16,666	+	+	+	+	—
1 20,000	+	+	+	+	+

TABLE 8

*Stability of subtilin-fast Staphylococcus aureus ATC 6538 that multiplied in a 1 2,000 concentration of subtilin in broth (table 1)*

CONCENTRATION OF SUBTILIN	SUBCULTURE			
	4th	10th	23rd	82nd
1 2,000	—	—	—	—
1 2,500	—	+	—	—
1 3,333	+	+	—	—
1 5,000	+	+	—	—
1 10,000	+	+	—	—
1 11,111	+	+	—	—
1 12,500	+	+	+	+

TABLE 9

*Stability of subtilin-fast Staphylococcus aureus ATC 6538 that multiplied in a 1 1,000 concentration of subtilin in broth (table 1)*

CONCENTRATION OF SUBTILIN	SUBCULTURE			
	4th	11th	17th	77th
1 1,000	+	—	—	—
1 1,111	+	—	+	—
1 1,250	+	—	+	—
1 1,428	+	—	+	—
1 1,666	+	—	+	—
1 2,000	+	+	+	—
1 2,500	+	+	+	+

*Subtilin-resistant Staphylococcus aureus* The parent strain of *Staphylococcus aureus* ATC 6538 multiplied in a 1 400,000 but not in a 1 300,000 concentration of subtilin. This strain became subtilin-resistant as shown in table 1. The

strain that grew in a 1 400,000 dilution of subtilin was transferred daily to nutrient broth and incubated at 37 C. At the end of 1, 5, 12, 18, 23, 29, and 89 days, portions of the cultures were removed and tested for their resistance to subtilin. This same procedure was followed using the remaining five resistant strains shown in table 1. The results are given in tables 4, 5, 6, 7, 8, and 9.

It may be seen that resistance to the antibiotic was retained even after as many as 91 serial transfers to broth. In general, the resistant strains tended to lose some of their fastness to subtilin, but in no instance did the resistance drop to that of the parent culture.

*Subtilin-resistant Mycobacterium phlei*. The parent strain of this acid-fast organism multiplied in a 1 500,000 but not in a 1 400,000 concentration of subtilin. This strain became subtilin-resistant as shown in table 2. The strain that

TABLE 10

*Stability of subtilin-fast Mycobacterium phlei that multiplied in a 1 2,000 concentration of subtilin (table 2)*

CONCENTRATION OF SUBTILIN	SUBCULTURE			
	3rd	9th	15th	72nd
1 1,666	—	—	—	—
1 2,000	+	+	—	—
1 2,500	+	+	—	—
1 3,333	+	+	—	—
1 5,000	+	+	—	—
1 10,000	+	+	—	—
1 11,111	+	+	—	—
1 12,500	+	+	—	—
1 14,285	+	+	+	—
1 16,666	+	+	+	—
1 20,000	+	+	+	—
1 25,000	+	+	+	—
1 33,333	+	+	+	+

grew in a 1 2,000 dilution of subtilin (ninth series) was transferred daily to nutrient broth and incubated at 37 C. At the end of 3, 9, 15, and 72 days, the cultures were tested for their resistance to subtilin. The results are given in table 10.

Resistance to the antibiotic was still pronounced after 72 daily transfers to broth. Although the resistant strain lost some of its fastness to subtilin, it did not drop to the same level as the parent culture.

## SUMMARY

*Staphylococcus aureus* (gram +), *Mycobacterium phlei* (acid-fast), and *Escherichia communior* (gram —), when exposed to sublethal concentrations of subtilin, readily developed a resistance or fastness to the antibiotic. This fastness was retained even after as many as 75 daily transfers to broth. In

general, the resistant strains tended to lose some of their fastness to subtilin, but in no instance did the resistance drop to that of the parent culture

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# A NEW TECHNIQUE FOR ISOLATING LISTERELLAE FROM THE BOVINE BRAIN

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It is relatively easy to isolate the causative agent from the brain in cases of ovine listerellosis. However, the direct isolation of the microorganism from the bovine brain has met with only partial success. This failure represents a challenge to microbiological technique. The investigation herein reported deals with a method whereby the percentage of direct isolations was increased.

Listerellosis in the sheep of Michigan has been reported in a previous publication (Gray *et al*, 1946). That it is present in this state in cattle also has been suspected for some time. Previous to this year a number of bovine brains had been submitted to the Animal Pathology Diagnostic Laboratory for culture when listerellosis was suspected, but it had never been confirmed by isolation of *Listerella monocytogenes*.

All cultures had been prepared by grinding the medulla in a mortar with about 10 ml of tryptose broth, then agitating with glass beads in a shaking machine for about 20 minutes. A portion (0.3 ml) of the resulting suspension was plated on tryptose agar and incubated at 37 C for 24 hours. The remainder was stored in the refrigerator at 4 C.

Case 1. On February 26, 1947, a bovine brain was submitted for culture. A clinical diagnosis of listerellosis had been made. Cultures were negative after 24 hours' incubation at 37 C, but when the brain suspension which had been stored in the refrigerator for 3 months was again plated, there was a heavy growth of listerellae (figure 1).

Case 2. March 24, 1947, a feeder steer was submitted for necropsy. This was the third animal in this herd to die. All three had displayed symptoms typical of listerellosis. Cultures prepared from the brain showed six colonies of a gram-positive organism resembling listerellae. Five weeks later when this same suspension was plated on tryptose agar, the colonies were too numerous to count.

Case 3. April 5, 1947, a six-month-old Hereford was presented for necropsy. This animal had been circling for about a week and was killed. Brain cultures

<sup>1</sup> Animal Pathology Section

<sup>2</sup> Bacteriology and Public Health Section

<sup>3</sup> Surgery and Medicine Section

<sup>4</sup> Approved by the Director of the Michigan Agricultural Experiment Station as Journal Article No. 916, n. s.

Part of a thesis to be submitted as partial fulfillment of the requirements for the degree of Master of Science, Department of Bacteriology and Public Health, Michigan State College. Approved by Ralph C. Huston, Dean, School of Graduate Studies.

were badly contaminated and no *Listerella* were observed. After refrigeration for 2 months it was possible to observe colonies of *Listerella* among the contaminants by the use of a dissecting microscope, as described by Huddleson (1946). The colonies of *Listerella* are a bright green with a finely textured surface. This is so characteristic that *Listerella* can be identified even in cases with extreme contamination.

Case 4 April 15, 1947, a yearling from a farm adjacent to that of case 2 was brought in for observation. It showed symptoms of listerellosis and died within 4 days. Brain cultures were negative. Three weeks later it was possible to demonstrate numerous colonies of *Listerella* from the refrigerated brain suspension.

Case 5 May 8, 1947, the brain of a two-year-old Holstein was presented for culture. A few colonies of a gram-positive rod were isolated (figure 2). With the suspension from this brain, an attempt was made to measure quantitatively the apparent increase in numbers of colonies observed in the preceding cases.

TABLE 1

Case 5 Showing increase in number of colonies during a period of approximately one month

	DATE				
	5-10	5-20	5-28	6-5	6-12
Plate I	90*	270	1,600	C	I
Plate II	—	220	1,400	C	I

\* Original plate C—Contamination I—Too numerous to count

A 0.2-ml portion of the brain suspension was plated on tryptose agar, a bent glass rod being used to distribute the material evenly. The results of this test appear in table 1.

In the five cases presented there was a marked increase in the number of *Listerella* that could be demonstrated after the brain suspension had been allowed to stand in the refrigerator for a period of time. There is as yet no definite explanation for this phenomenon, but several possibilities exist. It was found that *Listerella* will grow quite readily at a temperature of 4 C. Inoculated (one 4-mm loop) tryptose agar slants containing about 0.5 ml nutrient broth at the base showed visible growth in the broth in 3 days, and in 7 days there was considerable growth on the slant when incubated at 4 C. There is also the possibility of further tissue disintegration, thus releasing the organisms from the cellular substance. Or the phenomenon may be explained by the presence of some unstable inhibitory substance in the brain tissue. Nutini and Lynch (1946a,b) have found a substance in an extract prepared from both the human and the bovine brain that is bactericidal to *Staphylococcus aureus*.

The evidence presented in this report strongly suggests the presence of a bacteriostatic factor for *Listerella monocytogenes* in the bovine brain. The presence of such substances in animal tissues may account for the specific im-

munity to infection inherent in certain tissues. Listerellosis as manifested in the ovine is extremely acute, generally, with a short fatal course. In the bovine the disease is less acute, and recoveries have been reported (Biester, 1941, Grahm, 1943). Pouden (1947) has reported one outbreak of an extremely acute nature, but that may be considered as atypical. Bacteriologically, primary isolation of listerellae from the ovine seldom results in failure, but in the bovine primary isolations are made in less than a third of the suspects cul-



FIG. 1. SHOWING HEAVY GROWTH OF *LISTERELLA* AFTER A BOVINE BRAIN SUSPENSION HAD BEEN STORED IN THE REFRIGERATOR FOR THREE MONTHS.

tured even though listerellosis is confirmed by histopathologic sections (compare figures 2 and 3). These observations are similar to those reported by Gifford and Jungherr (1947). Cultures recently isolated from the ovine will produce a conjunctivitis in rabbits in about 18 hours, whereas those recently isolated from the bovine require 72 hours or more to produce a conjunctivitis. In general, cultures of a bovine origin show a latent pathogenicity as compared to the ovine strains.

*Biochemical tests.* All media and procedures were in accordance with those described in *The Manual of Methods for the Pure Culture Study of Bacteria*

Indole was not produced, nitrates were not reduced, litmus milk was reduced at first and after about 10 days showed a slight acid reaction,  $H_2S$  was produced in 24 hours, a slight zone of beta hemolysis appeared on blood agar, and starch was not hydrolyzed. The results of the fermentation reactions are shown in table 2.

*Animal inoculation* Two drops of a 24-hour agar slant growth of the culture from case 2, suspended in 5 ml broth and instilled in the conjunctival sac of an adult rabbit, produced only a slight reddening of the lids in 5 days of observation. This same animal was then given 0.5 ml intravenously of a similar suspension.

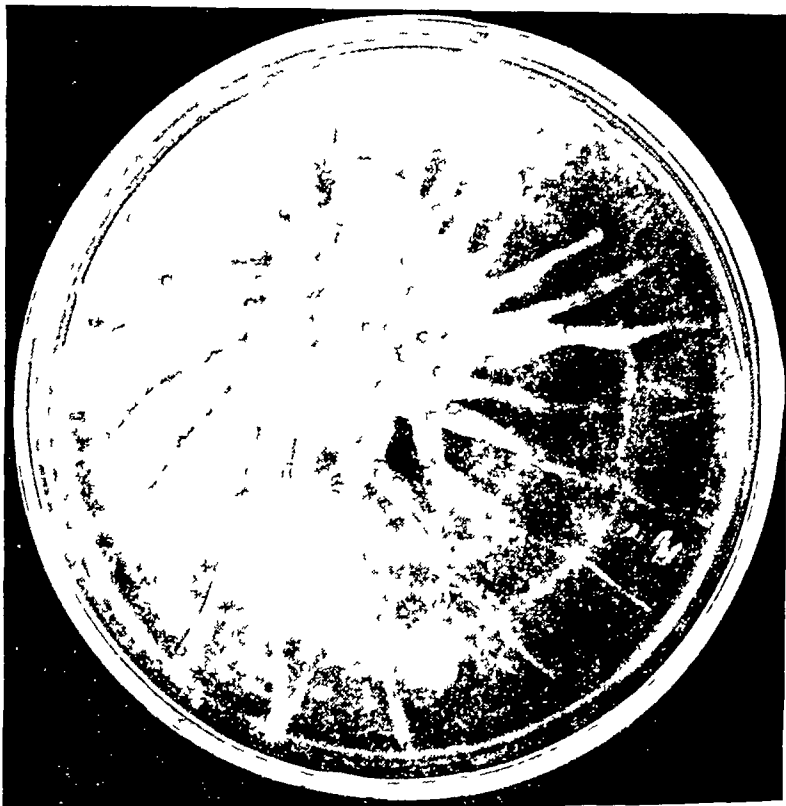


FIG 2 PRIMARY CULTURE OF BOVINE BRAIN CASE 5

The animal died in 48 hours. No gross lesions other than slight degeneration in the liver were observed. *Listerellae* were isolated from the heart blood and liver.

Two drops of a similar suspension of culture from case 4 were instilled into a rabbit's eye. After 96 hours the eye was slightly congested. This condition persisted for 8 days. At this time (the eighth day) *Listerellae* could not be isolated from the eye. On the ninth day the eye became more congested, and on the tenth day a severe conjunctivitis and keratitis were present. This persisted for 3 days. Swabs taken from the eye during this time when plated on tryptose agar showed a heavy growth of *Listerellae*. When the conjunctivitis cleared, the

animal appeared very ill. It was depressed, did not eat, and drank large quantities of water. No temperature was taken, but the ears were very hot to the

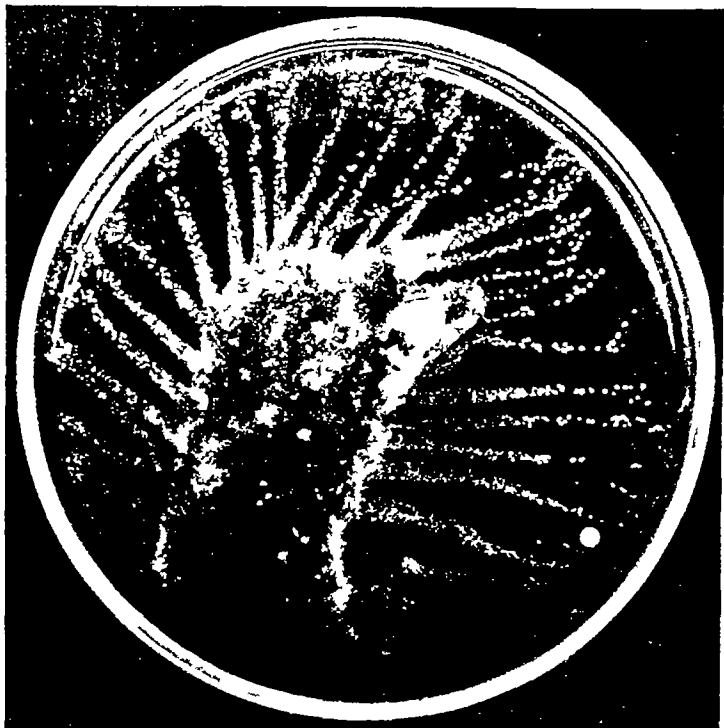


FIG. 3. TYPICAL PRIMARY CULTURE OF OVINE BRAIN. This animal was from the same farm as case 3 of this report.

TABLE 2  
*Fermentation reactions*

CULTURE	GLUCOSE	GALACTOSE	FRUCTOSE	ARABINOSYL	XYLOSE	LACTOSE	MALTOSE	SUCROSE	TRIPHALOSYL	MANNITOL	DENTRIN	INULIN	INOSITOL	DULCITOL	MANNITOL	SORBITOL	GLYCEROL	SALICIN
1	+	-	+	-	-	±	+	+	+	-	+	-	-	-	-	-	±	+
2	+	-	+	-	-	±	+	±	+	-	+	-	-	-	-	-	±	±
3	+	-	+	-	-	±	+	+	+	-	+	-	-	-	-	-	±	±
4	+	-	+	-	-	±	+	±	+	-	-	-	-	-	-	-	±	+
5	+	-	+	-	-	-	+	±	+	-	+	-	-	-	-	-	±	+

+—acid, ±—marked acid, ±—slight acid, ——no acid  
No gas was produced in any of the media

touch. It showed all the symptoms of listerellosis. They persisted for 48 hours, at the end of which time the animal was comatose, and was killed for necropsy. There were no gross lesions other than a slight degeneration of the

liver The heart blood, liver, and medulla were cultured on tryptose agar *Listerellae* were isolated from the medulla only Paraffin sections of the medulla showed the typical paravascular cuffing found in listerellosis

A similar suspension of a culture isolated from a sheep, which was brought in from the same farm as case 3 of this report, produced a marked conjunctivitis and keratitis in the eye of an adult rabbit in 24 hours This condition persisted for 10 days *Listerellae* were isolated from the eye for 12 days following instillation

#### SUMMARY

Five cases of listerellosis in the bovine have been reported and confirmed by laboratory study In three of the five cases isolations were made after the brain suspension had been refrigerated for from 5 weeks to 3 months A preliminary report is made relative to some substance in the bovine brain which may interfere with the primary isolation of *Listerellae*, but which may be destroyed by refrigeration A method of readily identifying colonies of *Listerella* is also described

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# THE OXIDATION OF AROMATIC COMPOUNDS BY FLUORESCENT PSEUDOMONADS

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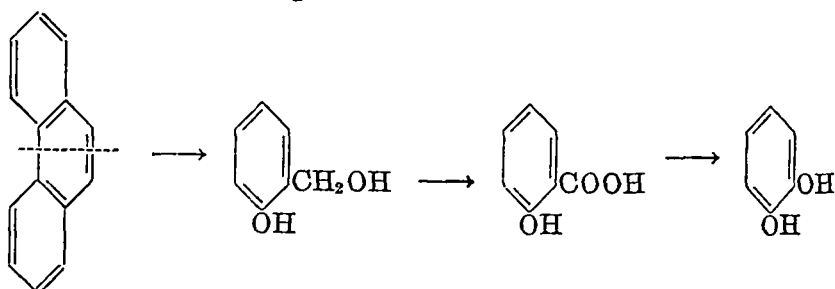
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Aromatic compounds are found among the cellular constituents of all organisms, and an understanding of the general mechanisms involved in their metabolism would be of great interest from the standpoint of comparative biochemistry. For research on the degradation of the benzene ring, bacteria appear at present to offer the most promising experimental material, since many of them are endowed with the ability to bring about a far-reaching oxidative attack on compounds of this class. This fact was first established by Wagner (1914), who found that such substances as phenol, cresol, phloroglucinol, and toluene can serve as sources of carbon and energy for soil bacteria. A number of subsequent investigators, notably Gray and Thornton (1928), have extended the list of simple aromatic compounds attacked and determined the nature of the microflora involved. It is now evident that many bacteria belonging to such diverse groups as the pseudomonads, aerobic sporeformers, mycobacteria, and actinomycetes can decompose and grow at the expense of aromatic compounds, provided that the initial substrate concentration is kept low to avoid toxic effects. In all cases so far known the attack is aerobic and strictly oxidative.

These bacterial dissimulations appear, at least in the majority of instances, to involve a rupture and destruction of the benzene nucleus itself, but our knowledge scarcely extends beyond this elementary fact. Only two workers have concerned themselves specifically with the biochemical aspects of the problem.

The first of these was Tausson, who made a series of investigations on the bacterial oxidation of aromatic hydrocarbons. Using appropriate enrichment procedures, he isolated specific bacteria capable of attacking naphthalene (1927), phenanthrene (1928), and toluene (1929), and in each instance investigated systematically, by means of growth in liquid synthetic media, the range and intensity of attack on related chemical substances. From the data thus obtained, he drew deductions concerning likely paths of breakdown, ruling out on the ground of nonutilizability certain chemically feasible intermediates and even, in a few cases, suggesting the probable biochemical mechanisms. Two instances may be summarized. The naphthalene-decomposing organism, *Bacterium naphthalinum*, was unable to attack the naphthols, diphenols and triphenols, and phthalic acid. Since he considered these substances to be the only likely products of a preliminary oxidation of one of the two rings in naphthalene, Tausson concluded that the attack by *B. naphthalinum* involved a simultaneous rupture of both the rings in the molecule, although he could obtain no evidence that this actually occurred. The specific organism attacking phenanthrene, of which three strains were tested for utilization of other aromatic compounds, showed

extreme selectivity, growing well only with saligenin, salicylic acid, and catechol, in addition to phenanthrene. From these results Tausson reasoned that there must be a direct relationship between the oxidation of these four compounds and postulated the following scheme



The only subsequent study on the dissimilation of aromatic compounds by bacteria was made by Bernheim (1940, 1941, 1942), who used manometric techniques to investigate the decomposition of benzoic acid and related substances by *Mycobacterium* spp. He concluded that benzoic acid is decomposed via one or another of the monohydroxybenzoic acids, but the evidence adduced in support of this contention is not convincing. The most interesting point brought out by his experiments is the extent of oxidative assimilation that accompanies these degradations. With a saprophytic mycobacterium, only four moles of carbon dioxide were formed per mole of benzoate oxidized, and since no organic end products of the oxidation were found outside the cells, it can be assumed that the three missing carbon atoms have been assimilated. Bernheim's data indicate further that the attack on aromatic compounds by mycobacteria is an adaptive one, although he himself did not draw this conclusion.

In view of the very scanty information at present available, it has seemed worth while to launch a fresh attack on the whole problem. As biological material, strains of the *Pseudomonas fluorescens* group have been used, this choice was dictated by den Dooren de Jong's demonstration (1926) that a number of simple aromatic compounds are included in the wide range of ternary substances that can serve as sole sources of carbon and energy for the growth of fluorescent pseudomonads, as well as by the ease with which these organisms can be handled. A few strains were obtained from other laboratories, and these were supplemented by a considerable number of fresh isolates from soil. The present paper comprises a summary of the preliminary results obtained.

#### MATERIALS AND METHODS

For growth experiments, 22 strains of fluorescent pseudomonads were used. Three of these were identified as *Pseudomonas pyocyanea*, one as *Pseudomonas chlororaphis*, and the remainder as members of the *P. fluorescens* species group, i.e., fluorescents producing no accessory pigments.

The ability to utilize aromatic compounds and related substances was determined in the first instance by growth experiments carried out according to the method of den Dooren de Jong (1926). A series of media were prepared con-



sisting of a mineral agar base (agar 2.0 per cent,  $\text{NH}_4\text{NO}_3$  0.1 per cent,  $\text{K}_2\text{HPO}_4$  0.1 per cent,  $\text{MgSO}_4$  0.05 per cent, pH 7.0 to 7.2) supplemented with the substances under test, a single specific organic compound being the sole added source of carbon and energy in each medium. A mineral agar medium with no added carbon source was included in the series as a control, since the impurities in agar itself permit a minimal amount of growth. Plates of these media were streaked with all 22 strains and incubated at 30°C, growth being recorded after 24, 48, and 72 hours. By comparing the development on plates containing added carbon compounds with that on the control plate, it is easy to determine whether or not utilization has occurred. The usual concentration of the organic compounds tested was 0.01 M, which is quite sufficient to yield good growth if utilization occurs, some of the more toxic compounds (e.g., phenol) were also used at a concentration of 0.005 M. Whenever most strains failed to develop on a given compound, the possibility that this was caused by toxicity was checked by incorporating the compound at the same concentration in yeast agar and determining whether or not the organisms were inhibited in their development on this medium. No such inhibition was found except with 0.01 M phenol, and hence it may be concluded that in the experiments to be reported the inability of a strain to grow with a given compound is a reflection of inability to metabolize it.

For manometric experiments the strains chosen were cultivated either on yeast extract agar (to produce cells unadapted to aromatic compounds) or on mineral agar of the composition given above supplemented by a single carbon source (to produce specifically adapted cells). After incubation at 30°C for 24 to 48 hours, the growth on such plates was harvested by washing them with M/60 phosphate buffer (pH 7.0), and following centrifugation the cells were resuspended in the same buffer. Two ml of such suspensions were used in each Warburg vessel. Gas exchanges were observed in an atmosphere of air at a temperature of 30°C.

*Possible methods for the analysis of the mechanisms of aromatic oxidations*  
A time-honored approach to the problem of detecting metabolic pathways is the determination, either manometrically or by growth experiments, of the utilizability of potential intermediates. As mentioned in the introduction, the work of Tausson on the oxidation of aromatic hydrocarbons was conducted entirely in this way. It has not always been realized that there are certain logical pitfalls in the interpretation of the data obtained from experiments on utilization, and since these pitfalls are well illustrated by Tausson's work, they are worth some discussion here. Tausson attempted to infer the early steps in the oxidation of phenanthrene from the utilizability as substrates for growth of other related aromatic compounds. He excluded such chemically feasible intermediates as phthalic acid on the ground of their immunity to attack, and with negative inferences of this sort there can hardly be any quarrel. The generalized form of the argument—"Although X is a chemically possible intermediate in the oxidation of A, its biological role is excluded by the fact that an organism capable of growing on A cannot grow on X"—is logically sound. But

this is by no means true of Tausson's *positive* inference that salgenin, salicylic acid, and catechol were intermediates in phenanthrene oxidation because they, too, were used as growth substrates by his phenanthrene-oxidizing bacterium. The ability to attack these substances may be coincidental, and by no means necessarily implies a sequential relationship. The general form of the hypoth-

TABLE 1

Utilization of benzoic acid and related disubstituted aromatic compounds as substrates for growth of fluorescent pseudomonads

ORGANISM AND STRAIN	BENZOATE	<i>o</i> -HYDROXYBENZOATE	<i>m</i> -HYDROXYBENZOATE	<i>p</i> -HYDROXYBENZOATE	<i>o</i> -PHTHALATE	<i>m</i> -PHTHALATE	<i>p</i> -PHTHALATE	<i>o</i> -TOLUATE	<i>m</i> -TOLUATE	<i>p</i> -TOLUATE	<i>p</i> -METHOXYBENZOATE	<i>p</i> -AMINOBENZOATE	HEXAHYDROBENZOATE
<i>P. fluorescens</i>													
A 3 1	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 2	+	0	0	+	0	0	0	0	(+)	0	0	0	0
A 3 3	+	0	0	+	0	0	0	0	(+)	0	0	0	0
A 3 5	+	0	0	+	0	0	0	0	(+)	0	0	0	0
A 3 6	+	0	0	+	0	0	0	0	(+)	0	0	0	0
A 3 7	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 8	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 9	+	+	+	+	0	0	0	0	(+)	0	0	0	0
A 3 10	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 12	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 13	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 14	+	0	0	+	0	0	0	0	(+)	0	0	0	0
A 3 16	0	0	0	+	0	0	0	0	0	0	0	0	0
A 3 17	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 18	0	0	0	+	0	(+)	0	0	0	0	0	0	0
A 3 19	+	0	0	0	0	0	0	0	0	0	0	0	0
A 3 20	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 29	+	0	0	+	0	0	0	0	0	0	0	0	0
<i>P. pyocyanea</i>													
A 3 15	+	0	0	+	0	(+)	0	0	0	0	0	0	0
A 3 27	+	0	0	+	0	0	0	0	0	0	0	0	0
A 3 28	+	0	0	+	0	0	0	0	0	0	0	0	0
<i>P. chlororaphis</i>													
A 3 30	+	0	0	+	0	0	0	0	0	0	0	0	0

0 = no growth, + = good growth, (+) = slight growth

esis is "From the chemical standpoint, B, C, D, and E represent possible intermediates in the oxidation of A, and since an organism that attacks A can also attack them, they are therefore actual intermediates." The weakness of the argument lies in the impossibility of deciding, from the available data, whether the relationship between A, B, C, D, and E is coincidental or sequential. This does not mean, however, that it is always impossible to draw positive inferences from experiments of this sort. The probability that the inferred relationship is

a sequential one can be greatly increased by working with a large number of related strains that differ in minor biochemical respects, provided one is willing to assume that the mechanism for the breakdown of A is the same in all these strains. If the ability to attack the five members of the series is coincidental, the chance of finding a strain that can attack A but not one of the other four substances increases with the number of strains tested, and only one instance of this behavior is needed to destroy the hypothesis of a sequence. On the other

TABLE 2

*Utilization of various aromatic substances, mostly monosubstituted, as substrates for growth of fluorescent pseudomonads*

SPECIES AND STRAIN	BENZOATE	BENZAL DEHYDE	BENZYL ALCOHOL	dl MAN DELATE	PHENYL- ACETATE	<i>p</i> -HY DROXY PHENYL- ACETATE	<i>β</i> PHEN YLPRO PIONATE	CINNA MATE	PHENOL
<i>P. fluorescens</i>									
A 3 1	+	+	+	0	+	0	0	0	0
A 3 2	+	+	+	0	+	0	0	0	+
A 3 3	+	+	+	0	+	+	0	0	0
A 3 5	+	+	+	0	+	+	0	0	0
A 3 6	+	+	+	0	+	+	0	0	0
A 3 7	+	+	+	0	+	+	0	0	0
A 3 8	+	+	+	+	+	+	0	0	+
A 3 9	+	+	+	0	+	0	0	0	+
A 3 10	+	+	+	+	+	+	0	0	0
A 3 12	+	+	+	+	+	+	0	0	0
A 3 13	+	+	0	0	+	+	0	0	0
A 3 14	+	+	+	0	+	+	0	0	0
A 3 16	0	0	0	0	+	+	0	0	0
A 3 17	+	+	+	0	0	0	0	0	0
A 3 18	0	0	0	0	(+)	+	(+)	(+)	0
A 3 19	+	+	0	0	0	0	0	0	0
A 3 20	+	+	0	0	0	0	0	0	0
A 3 29	+	+	+	(+)	0	+	0	0	0
<i>P. pyocyanea</i>									
A 3 15	+	+	+	(+)	0	+	0	0	0
A 3 27	+	+	+	(+)	0	+	0	0	0
A 3 28	+	+	+	(+)	0	+	0	0	0
<i>P. chlororaphis</i>									
A 3 30	+	+	+	(+)	0	+	0	0	0

0 = no growth, + = good growth, (+) = slight growth

hand, if the relationship really is sequential, any strain unable to attack one member of the series should be equally unable to attack the preceding members, and the discovery of this pattern of biochemical behavior provides powerful additional evidence of the correctness of the hypothesis. Experiments on utilization can never provide conclusive evidence for the existence of a biochemical sequence, but by conducting them with many strains, the probability that a sequence exists can be greatly strengthened. The danger of inferring sequential relationships from a combination of chemical possibilities and data on utilization by a few

strains can be clearly shown from the present work. The compounds phenyl acetate, mandelate, benzaldehyde, benzoate, and *p*-hydroxybenzoate comprise a chemically possible oxidative reaction chain, and as can be seen from tables 1 and 2, there are certain strains that can attack them all. Had these particular strains been the only ones employed in the experiments on growth, a sequential relationship between the five compounds could have been inferred, but other strains can attack phenylacetate without attacking other members of the postulated chain, and these patterns of biochemical behavior show clearly that the sequential relationship does not in this case exist.

A second method of studying the pathways of aromatic decompositions, namely, by the analysis of adaptive behavior, was discovered during the course of this work and has already been described (Stanier, 1947). The combined use of both approaches has proved most valuable, and the inferences drawn from growth experiments have checked precisely with those drawn from experiments on adaptation.

### RESULTS

*The utilization of benzoic acid and its derivatives.* As can be seen from table 1, benzoate is widely utilized by fluorescent pseudomonads as a substrate for growth, only 2 of the 22 strains tested being unable to attack it. On the other hand, substitutions in the benzoic acid ring as a rule cause a marked reduction in biological availability. Of all the compounds tested, the only substituted benzoate that is attacked by a majority of strains is *p*-hydroxybenzoate. This might suggest that *p*-hydroxybenzoate is an intermediate in the oxidation of benzoate, a possibility advanced by Bernheim (1942) in the case of mycobacteria. However, the behavior of strain A 3 19, which has the biochemical pattern benzoate +, *p*-hydroxybenzoate -, indicates that this is not so, and the same conclusion can be drawn from experiments previously reported (Stanier, 1947) on adaptation to the two compounds by strains that attack them both.

The very marked biological influence of positional substitution in the benzoate ring is illustrated by the results with the *ortho*- and *meta*-hydroxybenzoates, which are attacked immediately by only one strain. A second strain (A 3 27) is capable of producing mutants after 5 to 7 days that will grow on the *meta*-hydroxy compound. The behavior of the strains tested with respect to the *ortho*- and *meta*-hydroxybenzoates shows that they too cannot be considered as intermediates in benzoate oxidation.

The introduction of a second carboxyl group into the benzoic acid molecule, irrespective of its position (*o*-, *m*-, and *p*-phthalate) makes it almost completely unattackable. Two strains can develop feebly on *m*-phthalate, but none can attack the *ortho* and *para* compounds. A similar result follows the introduction of a methyl group (*o*-, *m*-, and *p*-toluate). *Meta*-toluate allows a scanty development by six strains, but the other two toluates are not utilized. There are indications that strains incapable of growing on *meta*-toluate may, nevertheless, cause a limited attack on this compound, the cells used as inoculum, and subsequently the surrounding agar, become brown on plates containing it. The nature

of this change has not been further investigated. In the hope of finding *para*-substituted benzoates other than the *p*-hydroxy compound that were generally attackable, *p*-methoxy- and *p*-aminobenzoate were also tested, but without effect.

The oxidizability of several substituted benzoates that were not used as substrates for growth was subsequently tested manometrically, but with negative results, the oxygen uptake by strain A 3 8 in the presence of *o*- and *m*-hydroxybenzoate, *o*-phthalate, *p*-methoxybenzoate, and *p*-aminobenzoate remains more or less at the autorespiratory level.

*The utilization of other aromatic compounds.* Among the other aromatic compounds tested, benzaldehyde, benzyl alcohol, phenylacetate, and *p*-hydroxyphenylacetate proved to be good substrates for the development of many strains. A few strains could also grow at the expense of mandelate, cinnamate,  $\beta$ -phenylpropionate, and phenol. The data on these compounds are given in table 2. The aromatic hydrocarbons, benzene, toluene, and xylene, were not attacked by any strains.

*The adaptive nature of the attack on aromatic compounds.* If a strain of *P. fluorescens*, after growth on yeast extract agar, is tested manometrically for oxygen uptake in the presence of aromatic substances that it can use as substrates for growth, there is invariably a marked time lag after substrate addition before oxygen consumption in excess of autorespiration becomes perceptible. Furthermore, the oxygen consumption in the presence of substrate, once initiated, does not proceed at a constant rate, but increases exponentially for some time until it reaches a fixed maximum, which is then maintained to the point of substrate exhaustion, this point being evidenced by a sharp break to the autorespiratory rate. A typical example of this behavior is shown in figure 1, representing the oxygen uptake with various aromatic compounds by *P. fluorescens* A 3 8 grown on yeast extract agar. The same type of behavior has been found with all strains tested after growth on this medium, and it occurs with all the aromatic compounds that these strains attack. Cells grown on a mineral medium with acetate as carbon source behave similarly. On the other hand, a strain that has been grown in a medium where a single aromatic compound is the sole source of carbon and energy, when tested manometrically for its ability to oxidize *that particular compound*, shows an immediate and rapid oxygen uptake after substrate addition that proceeds at a fixed rate to the point of substrate exhaustion. These findings show that the primary attack on aromatic compounds by fluorescent pseudomonads is in all cases brought about by adaptive enzymes, which are formed only when the cells are grown at the expense of an aromatic substance or when resting cell suspensions are placed in contact with a potentially oxidizable aromatic substance.

The time required for adaptation to occur (judged by the time after substrate addition to unadapted cells during which the oxygen uptake remains at the autorespiratory level) is generally of the order of 30 to 50 minutes, and never greater than 80 minutes under the conditions of our experiments. It varied slightly from one experiment to the next, doubtless as a result of variations in the age and

density of cell suspensions. The adaptation to *p*-hydroxybenzoate is intrinsically more rapid than to the other aromatic substrates tested, becoming perceptible with cells grown on yeast extract about 10 to 20 minutes before the first signs of adaptation to benzoate or phenylacetate. It should be pointed out that the adaptive process with fluorescent pseudomonads does not require the presence of an extraneous nitrogen source, all experiments have been carried out using well-washed cell suspensions in phosphate buffer.

The question naturally arises whether or not adaptation to one aromatic compound will cause adaptation to other members of the same class of com-

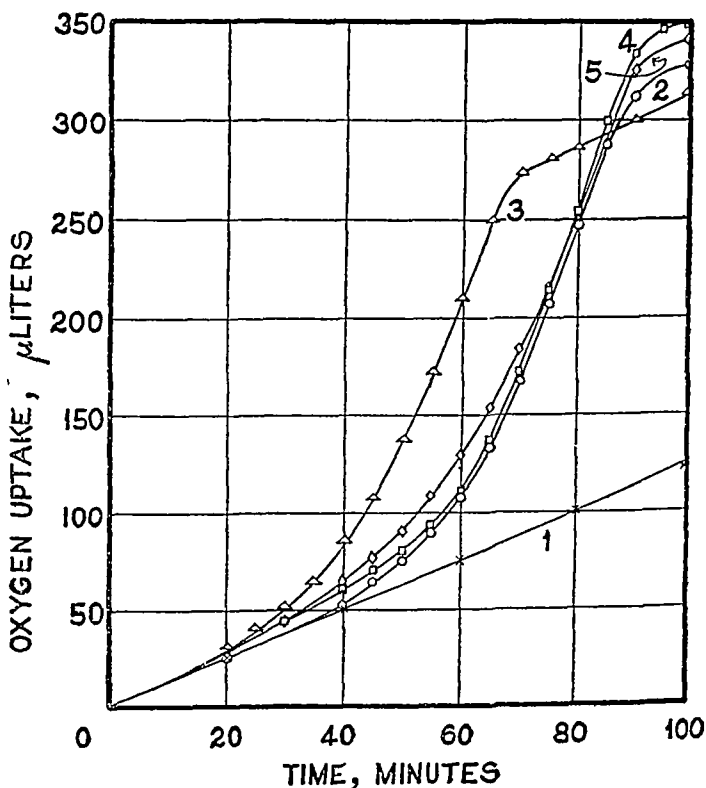


FIG 1 ADAPTIVE OXIDATION OF AROMATIC COMPOUNDS BY STRAIN A 38 GROWN ON YEAST EXTRACT AGAR

1 = autorespiration, 2 = 2 micromoles of benzoate, 3 = 2 micromoles of *p*-hydroxybenzoate, 4 = 2 micromoles of *dl*-mandelate, 5 = 2 micromoles of phenylacetate

pounds. This point has already been discussed in a previous paper (Stanier, 1947), in which it was shown that adaptation is a highly specific process and that complete, simultaneous adaptation to two or more aromatic compounds, after growth on one of them, occurs only when these compounds are members of a single reaction chain. One further example of the specificity of adaptation to aromatic compounds may be given here. *P. fluorescens* A 39 can grow at the expense of the disubstituted compound *p*-hydroxybenzoate and the two related monosubstituted compounds benzoate and phenol. It might accordingly be expected that adaptation to the compound containing both substituent groups

would result in simultaneous adaptation to at least one of the two compounds containing one of these substituent groups. This is not the case, as shown in figure 2, whereas *p*-hydroxybenzoate is attacked immediately at a steady rate by cells grown in its presence, benzoate and phenol are attacked by the same cell suspension only after a typical adaptive lag. This is the most clear-cut instance of adaptive specificity yet encountered and lends powerful support to the contention that complete, simultaneous adaptation is always a reflection of membership in a common metabolic pathway.

*Oxidative reactions proceeding through benzoate.* There are three substances related to benzoate—mandelate, benzyl alcohol, and benzaldehyde—whose patterns of utilization, as shown in table 2, indicate that they are decomposed via

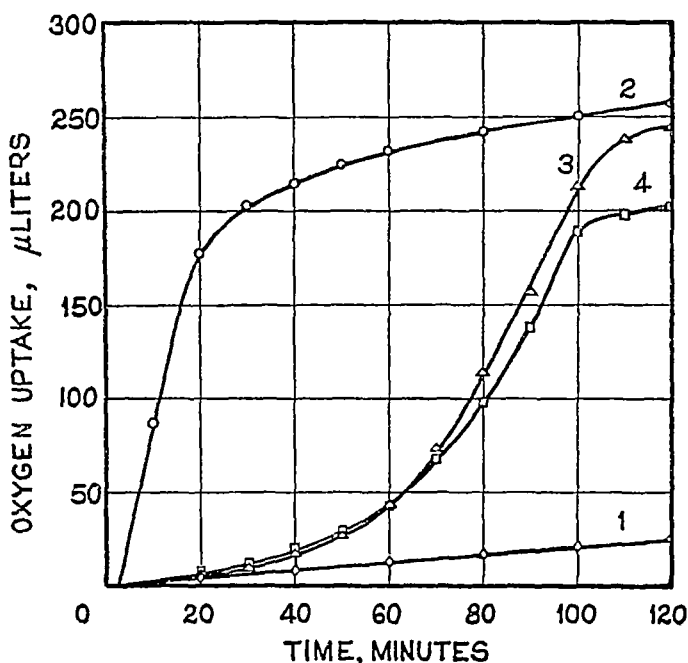


FIG. 2. OXIDATIONS BY STRAIN A 39 GROWN ON *p*-HYDROXYBENZOATE AGAR. 1 = autorepiration, 2 = 2 micromoles of *p* hydroxybenzoate, 3 = 2 micromoles of benzoate, 4 = 2 micromoles of phenol.

benzoate. The growth of strains on benzoate and benzaldehyde runs completely parallel, all strains capable of attacking one also possessing the ability to attack the other. This is to be expected from the experiments previously reported on the adaptive behavior of benzoate-grown cells, which show complete simultaneous adaptation for benzaldehyde. The number of strains that can attack benzyl alcohol and mandelate is fewer than the number attacking benzoate and benzaldehyde, but whenever an attack on the two former occurs the two latter are also attacked. This suggests that the three compounds mandelate, benzaldehyde, and benzyl alcohol are all decomposed via benzoate. Experiments on simultaneous adaptation (Stanier, 1947) substantiate the existence of the mandelate-benzaldehyde-benzoate chain. The participation of benzyl alcohol has not



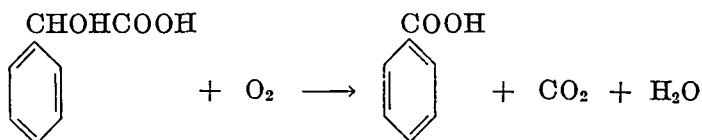


So far, no other reaction chains can be formulated for the oxidations of aromatic compounds by fluorescent pseudomonads. The evidence from growth experiments, summarized in tables 1 and 2, shows very clearly that certain chemically plausible sequences do not, in fact, occur. Thus phenylacetate cannot be oxidized either via the mandelate-benzoate reaction chain or via *p*-hydroxyphenylacetate, since there are a number of strains capable of growing with phenylacetate that cannot develop at the expense of these possible intermediates. The nonoccurrence of the mandelate-benzoate chain in phenylacetate oxidation can also be inferred from experiments on simultaneous adaptation.

Other compounds attacked by some strains whose utilization appears uncorrelated are phenol, *p*-hydroxybenzoate, and cinnamate and  $\beta$ -phenylpropionate. It thus appears that there are a considerable number of distinct primary oxidations of aromatic compounds that can be effected by fluorescent pseudomonads. It is, of course, possible that all these primary oxidations merge at some point in a common pathway, but until more is known about the attack on the benzene nucleus itself, this point will remain obscure.

*Oxidation of the stereoisomers of mandelate* It was mentioned in an earlier paper (Stanier, 1947) that both isomers of mandelic acid are attacked at the same rate by strains of *P. fluorescens* that can oxidize this compound. The pure isomers have not been obtainable for direct tests, but this conclusion can be safely drawn from manometric data with the racemic mixture.

Benzoate is an intermediate in the oxidation of mandelate, and therefore the total oxygen uptake per mole of mandelate oxidized should be equal to the total oxygen uptake per mole of benzoate oxidized plus the oxygen uptake required for the intermediate steps, which is one mole per mole.



From this it follows that if both isomers of mandelate are attacked, the total oxygen uptake per mole of *dl*-mandelate supplied will be greater by one mole than the total oxygen uptake per mole of benzoate, whereas if only one isomer is attackable it will be a little over half as great. The data from several experiments, summarized in table 3, show that within the limits of experimental error the total oxygen uptake with *dl*-mandelate is always greater by one mole per mole than the total oxygen uptake with an equivalent molarity of benzoate. Therefore both isomers must be attacked.

If the two isomers were attacked at different rates, the oxygen uptake with *dl*-mandelate by mandelate-adapted cells should show a change of rate prior to the final break resulting from substrate exhaustion, the change of rate occurring at the point where the more rapidly attacked isomer is completely used up. No sign of this has ever been found, the rate remaining steady from the point of substrate addition until the curve breaks once more to the autorespiratory level.

*Possible mechanisms for the attack on the benzene nucleus* The ability of fluo-

rescent pseudomonads to use simply substituted aromatic compounds like benzoate, *p*-hydroxybenzoate, and phenol as substrates for growth is prima facie evidence that these compounds undergo a far-reaching attack. This conclusion is substantiated by studies on carbon dioxide production during the oxidation of benzoate, which have shown that between 4 and 4.5 moles of CO<sub>2</sub> are produced per mole of benzoate oxidized. One is thus confronted with the problem of how a rupture and oxidation of the benzene nucleus can be brought about as the result of a series of simple step reactions. It is clear that none of the hydroxybenzoates are intermediates in the oxidation of benzoate, but beyond this no direct indications exist. On paper, only two possibilities appear reasonable.

The ring might be opened by oxidative rupture, without prior saturation of the double bonds, to yield an aliphatic unsaturated compound with a *cis cis* configuration and, probably, terminal carboxyl groups. The work of Jaffé (1909) showed that in the animal organism muconic acid can be recovered after the injection of benzene, providing a certain biochemical precedent for the con-

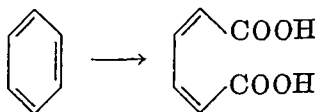
TABLE 3

*Relative total uptakes of oxygen, uncorrected for autorespiration, with 2 micromoles of benzoate and of dl-mandelate*

EXPERIMENT	TOTAL UPTAKE OF OXYGEN MICROLITERS		DIFFERENCE MICROLITERS	DIFFERENCE MOLES PER MOLE
	<i>dl</i> Mandelate	Benzoate		
1	282	232	50	1.11
2	269	230	39	0.87
3	284	246	38	0.86
4	326	284	42	0.94
5	298	256	42	0.94

Average excess uptake of oxygen with *dl*-mandelate over that with benzoate 0.94 moles per mole

sideration of this possibility. However, subsequent investigations (Drummond and Finar, 1938) have shown that the muconic acid produced in the animal organism is the *trans-trans* form, rather than the *cis-cis* form that might be expected to result from a direct oxidation of the benzene molecule.



and hence there is considerable uncertainty about its origin and relation to benzene oxidation. On more general grounds, however, an oxidative rupture of the type pictured above appears unlikely, since it presumably involves a direct dehydrogenation of the benzene ring, which is hard to envisage. A much more attractive concept is the possibility that oxidative attack on the ring may be preceded by a partial or complete saturation of the double bonds by water addition, a process that would at once destroy the resonance to which the benzene

ring owes its stability and allow dehydrogenations to occur. If this represents the first stage of attack on the ring, it should result in the production of hydroxy derivatives of cyclohexanol, or of compounds with a ring structure intermediate between cyclohexanol and benzene. There is a certain amount of indirect evidence that suggests the possibility of transformations along these lines in living organisms. Hydroxy derivatives of the cyclohexane series (quercitol, inositol), some of them containing carboxyl groups (quinic, shikimic, and sedanonic acids), are known to be widely distributed among plants. Furthermore, some of them (e.g., shikimic and sedanonic acids) have a double bond between two of the carbon atoms in the ring, thus being transitional chemically to the aromatic group. Hall (1937) and Dangschat and Fischer (1938) have postulated dynamic interrelationships in plants between cyclohexane and benzene derivatives.

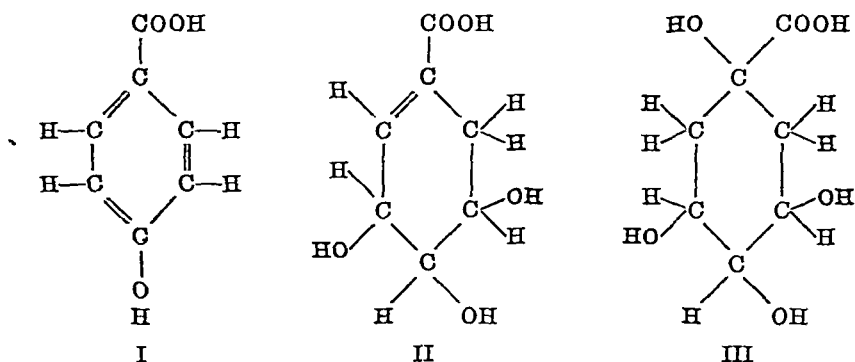


FIG. 4. THE FORMULAE FOR *p*-HYDROXYBENZOIC (I), SHIKIMIC (II), AND QUINIC (III) ACIDS, DRAWN TO INDICATE THE POSSIBLE MANNER IN WHICH ADDITION OF WATER TO THE *p*-HYDROXYBENZOIC ACID MOLECULE COULD OCCUR.

Insofar as the oxidation of benzoic and *p*-hydroxybenzoic acid is concerned, it is the hydroxycyclohexanecarboxylic acids, such as quinic and shikimic, that must be considered as possible intermediates. A comparison of the formulae for *p*-hydroxybenzoic, quinic, and shikimic acids shows the very close relationship that exists between them, theoretically *p*-hydroxybenzoic acid could be transformed into shikimic or quinic acid by the addition of two or three water molecules respectively, as is shown in figure 4. A decomposition of benzoic acid along much the same pathway is also possible and would involve only one dehydrogenation following water addition, an assumption that is compatible with the finding that it is not decomposed via *p*-hydroxybenzoic acid. There is another reason for considering a decomposition of aromatic acids through hydroxycyclohexanecarboxylic acids as a very attractive possibility. It is known that quinic and shikimic acids can be oxidized by chemical means to citric and *trans*-aconitic acids, respectively, through rupture of the ring between the third and fifth carbon atoms and the elimination of carbon dioxide (Fischer and Dangschat, 1934, 1935). If an oxidation of this type occurred biologically, a small number of simple step reactions would suffice to transform the aromatic acids into com-

pounds that could then be further attacked through the Krebs cycle. Reverse reactions along this path would also provide a possible mechanism for synthesis of the benzene ring.

Since quinic acid was commercially available, its possible intermediate role was tested. Growth experiments showed that it was readily utilized by 21 out of the 22 strains employed, thus constituting one of the most widely attacked ternary compounds for fluorescent pseudomonads. However, the single strain that was unable to use it as a growth substrate developed well with both benzoate and *p*-hydroxybenzoate. The possibility that quinic acid itself is an intermediate

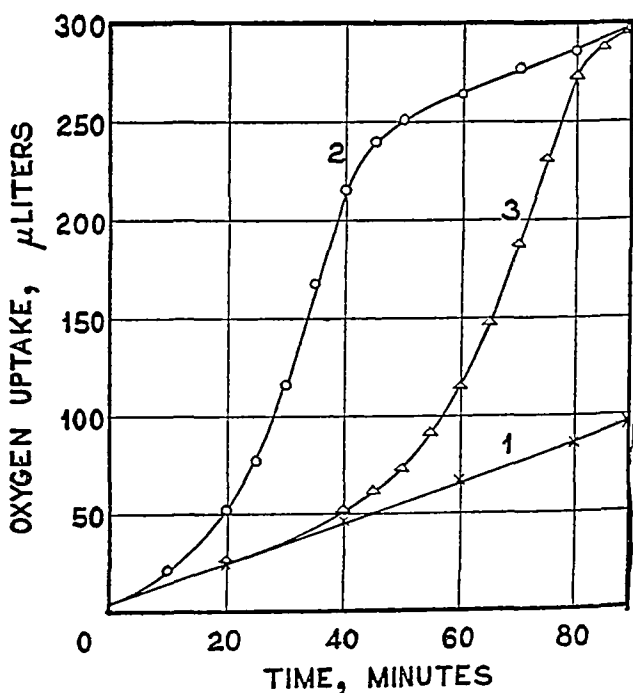


FIG 5 OXIDATION OF QUINATE AND BENZOATE BY STRAIN A 3 8 GROWN ON YEAST EXTRACT AGAR

1 = autorespiration, 2 = 2 micromoles of quinate, 3 = 2 micromoles of benzoate

in the oxidation of these compounds was then completely eliminated by adaptation experiments, using strain A 3 8, which can attack phenylacetate, benzoate, *p*-hydroxybenzoate, and quinate. In spite of their negative outcome, these experiments are perhaps worth recording as an illustration of the usefulness of the adaptive technique.

The oxidation of quinate by strain A 3 8 is adaptive, but adaptation occurs with much greater rapidity than in the case of aromatic compounds. During the first 10 minutes after substrate addition to cells grown on yeast extract there is an oxygen uptake appreciably greater than the autorespiration, and the steady maximum rate is very quickly attained. The relative rate of adaptation to benzoate and to quinate is shown in figure 5. It might almost be thought that

the attack on quinate is constitutive, were it not for the fact that the slight lag shown in figure 5 is completely abolished by growth in the presence of quinate (figure 7)

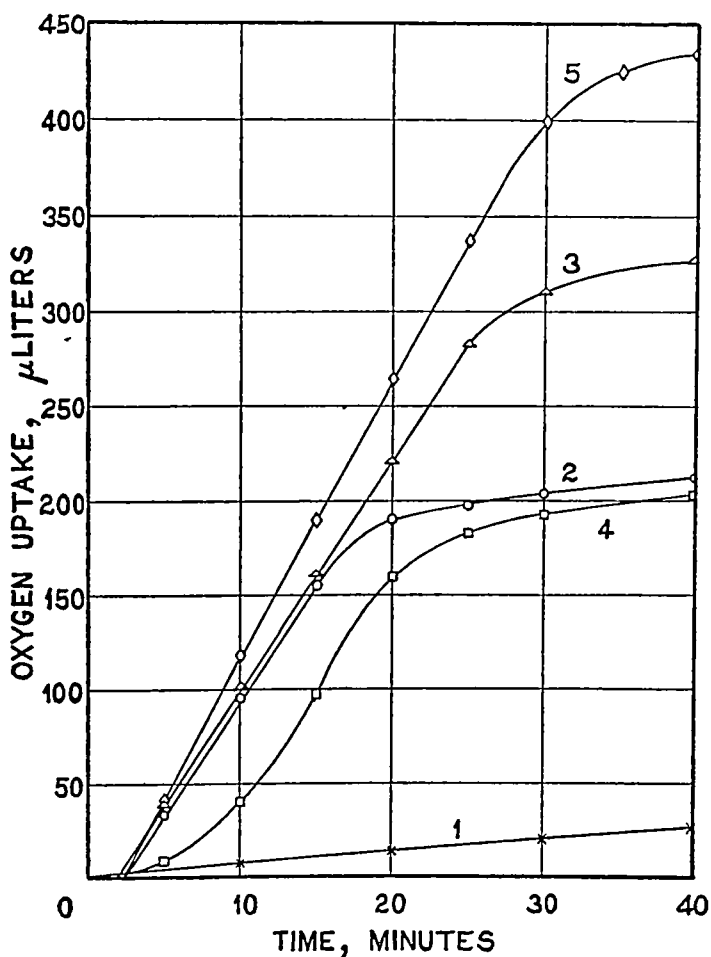


FIG 6 OXIDATION OF *p* HYDROXYBENZOATE AND QUINATE BY STRAIN A 38  
GROWN ON *p* HYDROXYBENZOATE AGAR

1 = autorespiration, 2 = 2 micromoles of *p*-hydroxybenzoate, 3 = 3 micromoles of *p*-hydroxybenzoate, 4 = 2 micromoles of quinate, 5 = 2 micromoles of quinate + 2 micromoles of *p*-hydroxybenzoate

The lag in quinate oxidation is not abolished by growth on phenylacetate, benzoate, or *p*-hydroxybenzoate, from which one may conclude that it is not an intermediate in the oxidation of any of these compounds. Furthermore, the rate of oxygen uptake by cells oxidizing *p*-hydroxybenzoate and quinate together is greater than the rate for *p*-hydroxybenzoate alone, although not quite additive. This indicates that additional dehydrogenating systems, not operative in

*p*-hydroxybenzoate oxidation, are brought into play when quinate is being simultaneously oxidized. The data illustrating these points are shown in figure 6.

Although it thus seems established that quinic acid is not an intermediate in the oxidation of the aromatic acids, the possibility remains that a related cyclohexanecarboxylic acid, with a slightly different arrangement of the hydroxy groups, or perhaps with one double bond in the ring, may belong in the reaction chain. Unfortunately no other compounds of this type could be obtained for testing purposes. One reason for suspecting that a compound of this type

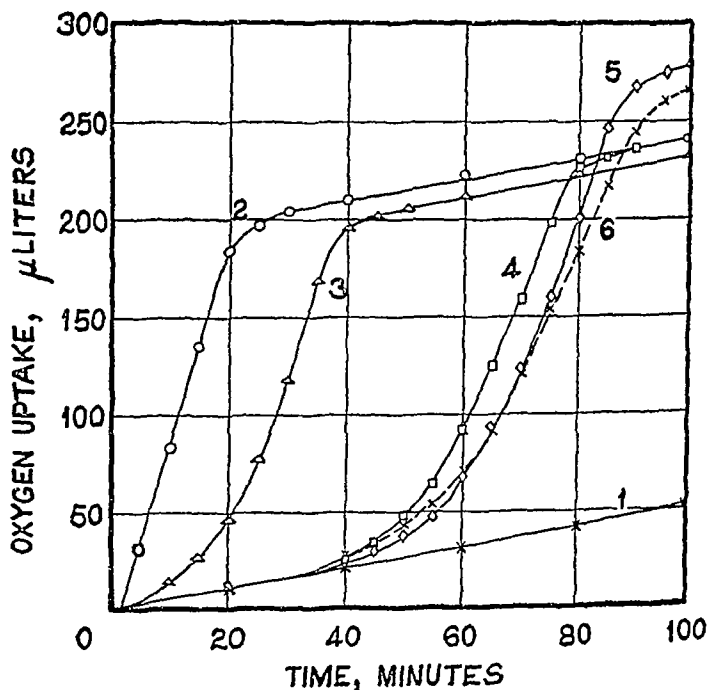


FIG 7 OXIDATION OF VARIOUS COMPOUNDS BY STRAIN A 38 GROWN ON QUINATE AGAR  
1 = autorepiration, 2 = 2 micromoles of quinate, 3 = 2 micromoles of *p*-hydroxybenzoate, 4 = 2 micromoles of benzoate, 5 = 2 micromoles of mandelate, 6 = 2 micromoles of phenylacetate

actually plays an intermediate role is the indication obtained from another experiment that there is an enzymatic interrelationship between the breakdown of quinate and of *p*-hydroxybenzoate. Cells grown on quinate show a striking and specific decrease in the time needed for subsequent adaptation to *p*-hydroxybenzoate, as can be seen from figure 7. This figure should be compared with figure 1, in which data on adaptation to aromatic acids by the same strain after growth on yeast extract agar are given. It can be seen that the time required for adaptation to benzoate, mandelate, and phenylacetate is very similar irrespective of whether the organism is grown on quinate or on yeast extract agar, whereas with *p*-hydroxybenzoate there is a marked difference.

## SUMMARY AND CONCLUSIONS

Fluorescent pseudomonads can attack many aromatic compounds and use them as substrates for growth. The attack is in all cases brought about by adaptive enzymes.

Adaptation to this class of compounds is extremely specific. Complete, simultaneous adaptation to two or more aromatic substances after exposure to one of them occurs only if the compounds in question comprise members of a common reaction chain.

Several lines of evidence indicate that *dl*-mandelic acid, benzoyl formic acid, benzaldehyde, and benzoic acid are successive members of a reaction chain. Benzyl alcohol also appears to participate in this chain (via benzaldehyde). In addition to this chain, there are several other distinct enzymatic mechanisms involved in the oxidation of aromatic substances by fluorescent pseudomonads, separate mechanisms existing for phenol, *p*-hydroxybenzoic acid, and phenylacetic acid.

The one optically active aromatic compound tested, mandelic acid, is attacked without discrimination between the two stereoisomers.

It is clear that these dissimulations involve a far-reaching degradation of the benzene nucleus. Possible alternative schemes whereby the initial stages of such a degradation might proceed are discussed. A potential intermediate in one of these schemes, quinic acid, is shown by various lines of evidence not to be functional in the dissimilation of aromatic acids. Certain data indicate, however, that a biochemical relationship exists between quinic and *p*-hydroxybenzoic acids, and it is therefore not excluded that some other hydroxycyclohexanecarboxylic acid may occur as an intermediate.

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# PHYSIOLOGICAL STUDIES ON SPORE GERMINATION, WITH SPECIAL REFERENCE TO CLOSTRIDIUM BOTULINUM<sup>1</sup>

## IV INHIBITION OF GERMINATION BY UNSATURATED C<sub>18</sub> FATTY ACIDS

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Unsaturated C<sub>18</sub> fatty acids, particularly oleic, are known to inhibit the growth of a great variety of bacteria (see Humfeld, 1947, for literature summary) In contrast, virtually nothing is known of the effect of such agents on the germination of spores preparatory to vegetative development Castor soap has been reported inhibitory to *Clostridium botulinum* (Gibbs *et al*, 1926), but it is not clear whether spores or vegetative cells or both were employed as inoculum

In a previous paper (Wynne and Foster, 1948a) we observed that incorporation of 0.1 per cent soluble starch in the spore-counting medium resulted in substantially higher counts on a pasteurized suspension of *C. botulinum* spores than non-starch control media, and furthermore that the characteristic dormancy of botulinum spores was eliminated under these conditions The starch evidently neutralizes some factor(s) inimical to germination of the spores Ley and Mueller (1946) studied a similar situation in which a factor present in ordinary agar was inhibitory to gonococci and also was neutralized by small amounts of starch The inhibitory material in agar had properties similar to oleic and stearic acids We have found that small amounts of oleic, linoleic, and linolenic acids inhibit the germination of *Clostridium* spores, that starch neutralizes this action, and that the presence of these acids in complex natural materials may account for the depressed germination rate and counts in the absence of starch

### EXPERIMENTAL PROCEDURE

General methodology, details of organisms, and background information may be obtained from the first paper of this series (Wynne and Foster, 1948a) The germination medium was generally Difco brain heart infusion broth with BBL thioglycolate supplement, and incubations were at 37 C in an atmosphere of natural gas (CH<sub>4</sub>) The counting medium was Yesair's pork infusion containing 0.1 per cent soluble starch The fatty acids (Na salts) were stored as 0.1 per cent aqueous solutions No loss in inhibitory effect of sodium oleate was observed over a storage period of 3½ months

*Comparison of various fatty acids* Table 1 shows that suppression of germination of *C. botulinum* 62A is proportional to the concentration of oleate added,

<sup>1</sup> This project has been undertaken in co-operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces The opinions or conclusions contained in this report are those of the authors They are not to be construed as necessarily reflecting the views or having the endorsement of the War Department

inhibition being practically complete with 10  $\mu\text{g}$  oleate per ml in a 24-hour incubation period, and already apparent with only 0.1  $\mu\text{g}$  per ml. Five other strains of *C. botulinum* (78A, 6B, 115B, 213B, and 29) show some degree of variation in susceptibility, but all are affected. Linoleic and linolenic acids are comparable to oleic acid.

Ten representative fatty acids in final concentrations of  $3.6 \times 10^{-6} \text{ M}$  ( $= 1 \mu\text{g}$  per ml oleate) were next compared for inhibitory action on botulinum germination. Those tested were pelargonic ( $\text{C}_9$ ), capric ( $\text{C}_{10}$ ), undecylenic ( $\text{C}_{11}$ ), lauric ( $\text{C}_{12}$ ), myristic ( $\text{C}_{14}$ ), palmitic ( $\text{C}_{16}$ ), stearic ( $\text{C}_{18}$ ), oleic ( $\text{C}_{18}$ ), linoleic ( $\text{C}_{18}$ ), linolenic ( $\text{C}_{18}$ ). Significant inhibition was exerted only by the three unsaturated  $\text{C}_{18}$  acids and palmitic ( $\text{C}_{16}$ ). Though palmitic acid repeatedly proved to be as effective as oleic at concentrations of 1  $\mu\text{g}$  per ml, its action did not increase significantly at higher concentrations as did that of the unsaturated  $\text{C}_{18}$  acids. Noteworthy was the inactivity of stearic acid, the saturated homolog of oleic, and also the unsaturated  $\text{C}_{11}$  acid, undecylenic, the well-known inhibitor of dermatophytic fungi. Stearic acid is devoid of inhibitory power even at a

TABLE 1  
*Oleic acid and germination of Clostridium botulinum spores*

OLEIC ACID	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION	INHIBITION
$\mu\text{g/ml}$	hours			%	%
—	0	530	—	—	—
—	24	120	410	77	—
0.1	24	240	290	55	29
0.5	24	360	170	32	59
1.0	24	400	130	25	68
10.0	24	520	10	2	97

concentration of 100  $\mu\text{g}$  per ml. Tests of various combinations of the unsaturated  $\text{C}_{18}$  acids failed to reveal any evidence for synergistic action in inhibiting germination. Linolenate actually proved to be substantially more effective than oleate (table 4).

*Complete suppression of germination.* With concentrations of 10  $\mu\text{g}$  per ml of any of the three unsaturated  $\text{C}_{18}$  fatty acids, neither measurable germination nor turbidity occurred during the usual short (about 24-hour) incubation periods. Such findings prompted a more prolonged study of this apparently complete inhibition of germination. Since a very few spores might conceivably germinate and produce turbidity through subsequent vegetative development (Wynne and Foster, 1948b), the nonappearance of turbidity would be the most sensitive criterion of full inhibition of germination, provided that it could be shown that vegetative cell development is less sensitive to oleic acid than spore germination. Furthermore, as shown below in media with higher concentrations of oleic acid, a sporocidal effect was observed over long incubation periods, thus rendering residual spore counts meaningless in such cases, and necessitating the adoption of turbidity as the criterion of 100 per cent inhibition.

Table 2 shows that oleic acid up to the 100- $\mu$ g level had a negligible effect on growth of spore-free vegetative cell inoculum except perhaps when the inoculum was insignificantly low, i.e., 3 to 30 cells per ml. The original inoculum count was made by direct microscopic count with a Petroff-Hauser chamber. To prevent killing the sensitive vegetative cells by exposure to oxygen during dilution freshly boiled medium was used.

In contrast, spores free of vegetative cells and tested in a manner similar to the vegetative cell experiment are decidedly sensitive to oleate inhibition (table 3).

TABLE 2

*Oleic acid and vegetative development of Clostridium botulinum 62A*

(Figures represent approximate average time of appearance of turbidity in hours)

OLEIC ACID  $\mu\text{g/ml}$	VEGETATIVE INOCULUM CELLS PER $\mu\text{L}$						
	3	30	300	3 000	30 000	300 000	3 000 000
0	22	19	16	14	<14	<14	<14
1	21	19	16	14	<14	<14	<14
10	25	24	19	18	16	<14	<14
100	(-) to 60*	(-)	34	26	19	<14	<14

(-) = no turbidity in 18 days

\* One of the duplicate tubes showed turbidity in the time specified, the other remained negative for the observation period.

TABLE 3

*Effect of oleic acid on spore germination in Clostridium botulinum 62A*

(Figures represent approximate average time of appearance of turbidity in hours)

OLEIC ACID  $\mu\text{g/ml}$	INOCULUM SPORES PER $\mu\text{L}$					
	5	50	500	5 000	50 000	500 000
0	46	46	33	25	20	20
1	40	33	37	25	20	18
10	(-)	(-)	(-)	(-)	31	26
100	(-)	(-)	(-)	(-)	(-)	(-)

(-) = no turbidity in 4½ months

No sign of germination was evident by microscopic examination of the spore tubes with 100  $\mu$ g oleate after 72 days' incubation. Failure to develop turbidity is, therefore, a valid criterion for complete suppression of germination.

Germination inhibition is in marked dependence on the medium used. In synthetic amino acid medium and also in a "casamino" acid medium, results were generally similar to those in table 3, but 10  $\mu$ g of oleate were somewhat less effective at spore levels of 500 and 5,000 per ml.

The inhibitory action of oleate was clearly of another order of magnitude when tested in a new (second) lot of Difco brain heart infusion. Linolenate was also

compared in this experiment (table 4) Both oleate and linolenate were strikingly less inhibitive to germination in the second lot (2) of Difco medium than in the first lot of the same medium As a matter of fact the interpretation of the experiment depends solely on which bottle of Difco medium the investigator chances to pull off the shelf In either case, however, linolenate proves to be a more potent inhibitor than oleate, by a factor of approximately threefold One-tenth per cent starch is not enough to detoxify 100  $\mu\text{g}$  of linolenate in either medium, but it is sufficient to detoxify 30  $\mu\text{g}$  linolenate in lot 2 medium

Recent experiments, to be reported later, have shown that neutral lipid substances also can neutralize the inhibitor action of oleate As prolonged extraction of the dehydrated medium of lot 1 in a Soxhlet with ether and with petroleum ether failed to alter materially the effect of added oleate, it is presumed

TABLE 4

*Oleate and linolenate inhibition of spore germination in two lots of Difco brain heart infusion*  
(Figures represent time of appearance of turbidity in hours)

Na OLEATE	Na LINOLENATE	STARCH 0.1 PER CENT	LOT 1	LOT 2
$\mu\text{g/ml}$	$\mu\text{g/ml}$			
0	0	—	24	20
3	0	—	36	22
10	0	—	(—)*	28
30	0	—	(—)	48
100	0	—	(—)	48
100	0	+	(—)	60
0	3	—	60	22
0	10	—	(—)	48
0	30	—	(—)	(—)
0	30	+	(—)	36
0	100	—	(—)	(—)
0	100	+	(—)	(—)

\* (—) = no turbidity by the fifth day

that the discrepancy between the two lots is due to their different contents of inhibitory substances, possibly fat acids Being present in the salt form, these were not extracted by the fat solvents

*Sporocidal action of oleic acid* Tubes containing 100  $\mu\text{g}$  per ml of oleic or linolenic acid and incubated 36 days have shown counts of only 0.5 to 2 per cent of the 550 incubated spores, with no turbidity occurring To eliminate the possibility of chance dormancy the counting tubes were reincubated for 26 days after the usual 3-day readings, but with no increase in colony count Zero time controls showed that the starch content of the medium effectively neutralized any fatty acid inhibition carried over in counting Direct tests with 0.1 per cent starch show complete neutralization of 10  $\mu\text{g}$  per ml of oleate—an amount in excess of that carried over into the counting medium One would conclude that 98 to 99.5 per cent of the inoculated spores were rendered nonviable through prolonged contact with oleate

But, again it is the medium that makes this conclusion highly suspect, for the same concentration of oleate has no sporocidal action over 36 days when the spores are inoculated into distilled water instead of medium. Under these conditions spore recovery was identical with and without the oleate.

Oleate per se is not, therefore, sporocidal. The only likely interpretation to explain the sporocidal experiment in the medium is that the medium itself is sporocidal, or that it lends to oleate a sporocidal action not evident with this substance itself. This action is not fast, for it does not show up at least for the first several days, i.e., during this period a true inhibition, not killing, is taking place. As vegetative cells themselves are resistant to oleate inhibition, it does not seem likely that the medium effect can be ascribed to a killing action of oleate on spores in any stage of germination that the medium would allow. It is evident that a sporocidal medium could be detected only under the condition

TABLE 5

*Effect of oleic acid on spore germination in other species of clostridia*  
(Figures represent approximate time of appearance of turbidity in hours)

OLEIC ACID	INOCULUM, SPORES PER ML				
	Putrefactive anaerobe no. 3679			<i>Clostridium chauvoei</i>	
	5 000	50 000	500 000	5 000	50 000
$\mu\text{g/ml}$					
0	12	<12	<12	12	10
1	12	<12	<12	12	10
10	24	17	12	13	10
		(-)	24*		
100	(-)	36*	(-)	38	36

(-) = no turbidity in  $4\frac{1}{2}$  months

\* One of the duplicate tubes showed turbidity in the time specified, the other remained negative for  $4\frac{1}{2}$  months

that prevailed here, namely, that the spores cannot germinate, as they cannot in the presence of oleate.

*Resistance of spores which show border-line germination.* The appearance of turbidity with a spore inoculum of 50,000 but not with 5,000 per ml in the presence of 10  $\mu\text{g}$  of oleate (table 3) might be hypothesized as being due to the presence of resistant spores such that 1 up to 10 were present in 50,000 but not 5,000. An analogous explanation has been postulated for resistance of *C. botulinum* spores to penicillin (Curran and Evans, 1946) and is a well-known event in drug resistance of bacteria. However, three such "border-line" spore tubes counted shortly after turbidity appeared gave an average of 93 per cent germination, thus contradicting that hypothesis.

Since the sporocidal action of the medium and oleic acid might have complicated these results, a different approach was adopted. Serial loop transfers of "border-line" tubes were made at 18- to 24-hour intervals in brain heart infusion broth containing 100  $\mu\text{g}$  per ml of oleic acid to eliminate ungerminated spores,

since it had been demonstrated that spore germination does not occur under such conditions. Two such cultures were then allowed to sporulate in the absence of oleate and were heated 20 minutes at 75 C to eliminate vegetative cells. A comparison of these spores with those of the parent strain failed to demonstrate a difference in oleate resistance, a fact rather suggestive that inherent spore variation and selection is not the explanation for the germination in the border line cases, although some other remote possibilities do exist.

*Other species* Four other anaerobic species were tested for spore germination inhibition with the technique given above for *C. botulinum* (table 5). Medium lot no. 1 was employed in this experiment. *Clostridium perfringens* and putrefactive anaerobe no. 3679 were similar, both being somewhat more resistant to oleate inhibition than *C. botulinum*. Microscopic examination of tubes showing no turbidity after 63 days' incubation revealed no signs that any germination had taken place. Spores of *Clostridium chauvei* and *Clostridium histolyticum* both were much more resistant to oleate, 100  $\mu$ g per ml being insufficient to suppress germination of a 5,000-spore inoculum.

With four species of aerobic spores, *Bacillus brevis*, *Bacillus megatherium*, *Bacillus mesentericus*, and *Bacillus subtilis*, no significant retardation of germination by 100  $\mu$ g per ml oleate was noted with any spore inoculum down to 5,000 spores per ml, the smallest number tested.

#### DISCUSSION

The inhibition by the unsaturated  $C_{18}$  fatty acids of spore germination in clostridia is unique because it is specific for the germination process, there being no significant inhibition of the vegetative cells after germination. This makes it possible to maintain and to study viable spores under conditions in which otherwise they would rapidly pass from the spore to the vegetative state, a feature which may be a handy tool in studying the metabolism of spores.

The strikingly different results obtained in different lots of the same commercial dehydrated medium indicate strongly that the investigator in bacteriology must not be misled into the innocent belief that commercial media are uniform or even satisfactory, merely because they are furnished dehydrated for convenience.

Part of these discrepancies doubtless may be ascribed to the content of inhibitory long chain fat acids. The presence of starch can overcome these inhibitory substances, and doubtless some others that may be present, and is thus a means of ensuring a minimum of inhibition from constituents of the medium itself.

#### SUMMARY

Oleic, linoleic, and linolenic acids have been shown to be strongly inhibitory to the germination of spores of six strains of *Clostridium botulinum*, linolenic being most effective, stearic acid was completely inactive. Spores of *Clostridium perfringens* and putrefactive anaerobe no. 3679 were less inhibited, whereas spores of *Clostridium histolyticum* and *Clostridium chauvei* were only slightly affected by oleate. Spores of four aerobic species were unaffected.

Oleic acid in a concentration of 100  $\mu$ g per ml prevented germination of large spore inocula of *C. botulinum* over 4½ months

Vegetative cells of *C. botulinum* are not inhibited by these acids

Oleate at 100  $\mu$ g per ml is not sporocidal itself, as killing was not observed in distilled water but only in a complete medium (brain-heart)

The degree of effectiveness of these  $C_{18}$  acids in suppressing germination was greatly different in two different lots of the same medium

The effect of inhibitory substances in nutritionally adequate media may cause more conflicting results in bacteriology than is now appreciated Starch (0.1 per cent) can neutralize fatty acid type inhibitors and possibly other types

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# THE RELATIONSHIP OF THE AGE OF THE BACTERIAL CULTURE TO THE DELAY IN SULFONAMIDE BACTERIOSTASIS

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The information on the influence of the age of the bacterial culture on the inhibition of bacteria by the sulfonamides is limited and contradictory. Fisher and Armstrong (1947), using as young cells a 16-hour culture of *Escherichia coli* and as old cells a 22-hour culture, found the young cells were more susceptible to the action of sulfathiazole. Chain *et al* (1945), Muir *et al* (1942), and Rose and Fox (1942) also believed young cells were more sensitive to the bacteriostatic action of the sulfonamides. Wolff and Julius (1939) reported that the age of the culture was of no importance in the mechanism of sulfonamide inhibition, but Long and Bliss (1939) and Finklestone-Sayliss *et al* (1937) indicated that old cells were more sensitive than young cells. Many investigators (Lockwood, 1938, White and Parker, 1938, Chandler and Janeway, 1939, Lockwood and Lynch, 1940, Spring *et al*, 1940, Lowell *et al*, 1941, Boroff *et al*, 1942, Muir *et al*, 1942, Brueckner, 1943, Chain *et al*, 1945) have reported that a delay of 1 to 6 hours occurs before the sulfonamides inhibit the growth of bacteria. Hirsch (1944) added "ngafen," a derivative of sulfanilamide, at various times during the growth of *E. coli* and staphylococci and found a delay in the bacteriostatic action of the drug irrespective of the point at which the drug was added. Wolff and Julius (1939) obtained similar results on adding sulfanilamide to hemolytic streptococci, and said it was necessary for bacteria to multiply a number of generations before the sulfanilamide could inhibit growth. Lowell, Strauss, and Finland (1941) added sulfapyridine at various times during the growth of a culture of pneumococci inoculated from an 8- to 10-hour growth, and obtained a lag in the action of sulfapyridine throughout.

However, a number of observations have been recorded (Libby, 1940, Hobby *et al*, 1942, Sevag and Shelburne, 1942) in which no delay in the antibacterial action of the sulfonamides occurred. Although many theories have been proposed to explain this puzzling phenomenon, no explanation to date seems adequate (Henry, 1944).

It has been noted by many (Clark and Ruehl, 1919, Henrici, 1922, Eaton, 1931, Martin, 1932, Walker *et al*, 1934, Huntington and Winslow, 1937, Hershey and Bronfenbrenner, 1938, Hershey, 1938) that, during bacterial growth, bacteria vary not only with regard to rate of growth but also physiologically. This is illustrated by the findings that young cells are more sensitive than old cells to such substances as bacteriophage (Bayne-Jones and Sandholzer, 1933), heat (Schultz and Ritz, 1910, Reichenbach, 1911, Sherman and Albus, 1923), cold (Sherman and Albus, 1923), and chemicals (Sherman and Albus, 1923, Watkins

and Winslow, 1932) Therefore, it seemed reasonable that bacteria during their growth might vary also in their sensitivity to the bacteriostatic action of the sulfonamides, and this might account for the discrepancy in the reports on the occurrence of a delay in the bacteriostatic action

In the present work the rate and amount of growth of *E coli* were determined in a synthetic medium and a nutrient broth by means of turbidity readings, plate counts, and measurement of cell size With this information it was possible to determine accurately the effect of sulfanilamide on cultures of any physiological or chronological age

#### METHODS

*Organism* *E coli* was chosen as the test organism since it grows well in a synthetic medium (Kohn and Harris, 1941, White *et al*, 1941, Cowles, 1942), is susceptible to the action of the sulfonamides (Kohn and Harris, 1941, Strauss *et al*, 1941, White *et al*, 1941), and does not secrete SA antagonists into the medium (Kohn and Harris, 1941, Lowell *et al*, 1941) A stock culture, *E coli*, number I, was used On plating out this organism, a rough colony phase was found, and both phases were subcultured The two cultures were streaked on plates at frequent intervals to verify the presence of only the smooth or of only the rough cultural phases To check the results with this strain, a second smooth culture strain was employed, the American Type Culture Collection strain 6522, the strain employed by Kohn and Harris (1941) Stock slants of these organisms were kept in the refrigerator at 5 C and were subcultured twice a month

*The size and age of the inoculum* Except as otherwise specified, the following conditions were maintained The inoculum for the tests consisted of cells grown in the medium to be used and washed twice by centrifugation with the same medium The size of the inoculum consisted of approximately 175 million organisms as determined by the viable plate count method, and was measured by the Evelyn photometer to give a density reading of 90 This inoculum was taken from a 16- to 18-hour culture In the synthetic medium, growth had stopped for the most part at around the sixth hour, so that the organisms used were, therefore, in a resting stage and were considered old cells To define young and old cells as used in this work, young cells were those cells present late in the lag phase and during the logarithmic phase of growth Old cells were those present during the early lag phase and at the end of the growth cycle This definition was based on the work of investigators who found that cells present in the late lag phase and progressing into the early phase of logarithmic growth increased in size (Clark and Ruehl, 1919), in respiratory activities (Walker *et al*, 1934), in metabolic activities (Bayne-Jones and Rhees, 1929, Walker and Winslow, 1932), and in rate of fermentation (Stark and Stark, 1929) These cellular activities began decreasing during logarithmic growth, so that at its termination, the bacteria resembled those present in the original inoculum Therefore, since these bacteria were in a resting stage, it was assumed they were old cells (Topley and Wilson, 1946)

Although growth had not completely stopped in the nutrient broth at the end of 16 to 18 hours, the bacterial growth curve obtained with an inoculum from a 16- to 18-hour culture and that obtained with an inoculum from a 64-hour culture in which growth had stopped were found to be identical except that the 64-hour culture grew at a slower rate. Therefore, an inoculum from a 16- to 18-hour culture in nutrient broth was used as it corresponded more closely to that from the synthetic medium. A 36-hour culture was used as an older culture from the synthetic medium, and for cultures younger than 16 hours, the sulfanilamide was added directly to the growing bacterial culture.

*Media* A synthetic medium (S-G) consisting of salts and glucose as recommended by Kohn and Harris (1941) was used. Water redistilled from glass was employed in the preparation of the S-G medium as bacterial growth did not occur regularly when ordinary distilled water was used. The nutrient broth recommended by Hershey (1939) was used as the other medium, and consisted of Difco peptone and beef extract in a phosphate buffer. Eighteen ml of the two media were added separately to the standardized Evelyn test tubes. The hydrogen ion concentrations of the media were adjusted to pH 7.2 with a Coleman electrometer.

*Glassware* All test tubes were cleaned by placing them in cleaning solution overnight. After being thoroughly rinsed with tap water and distilled water, they had to be rinsed also with redistilled water in order to obtain bacterial growth consistently. They were sterilized by autoclaving at 20 pounds for 20 minutes.

*Turbidity readings* Many investigators (Libby, 1940, Kohn and Harris, 1941, MacLeod and Mirich, 1942) have used this method for measuring bacterial growth. Hershey (1939) found that density readings are an accurate method for the determination of growth since nitrogen determinations were proportional to the turbidity readings. Turbidity readings also agree with the viable plate count method if it is remembered that the increase in density during the lag phase of growth may be due to an increase in cell length and not to an increase in cell population. Filter no. 520 was used in the Evelyn photometer as recommended by Kohn and Harris (1941).

*Viable plate count method* The method employed as a modification of the one used by MacLeod and Mirich (1942). A wire transfer loop which contained at room temperature a volume of 0.007 ml was used to make the first dilution in saline from the test material. A separate pipette was used for each of the two higher dilutions. Two sets of dilutions were made from each culture, and 3 pour plates were made from the last dilution, thus making a set of 6 plates from each culture. The error was approximately plus or minus 10 per cent. The plates were made at the same times as the turbidity readings, so that a correlation could be made between the two. The plates were incubated at 37 C for 36 hours and then counted.

*Measurement of cell size* Measurements of cell length were made from direct smears which had been stained by gentian violet and were measured by using a micrometer and a no. 10 ocular in the microscope. Although cell size is smaller

than its real value when measured in this fashion, as pointed out by Knaysi (1944) and Huntington and Winslow (1937), the relationship between the sizes is of like degree

**Sulfanilamide concentrations** The routine SA concentrations used for each medium retarded the growth of *E. coli* approximately one-third when added with the inoculum. For the synthetic medium this SA concentration was 10 mg per cent, and for the nutrient broth 750 mg per cent. The cultures were grown in a constant temperature water bath at 37.5°C.

### RESULTS

**Normal growth curves in the two types of media** To determine the effect of SA on the growth of bacteria in different media, it was necessary to ascertain first

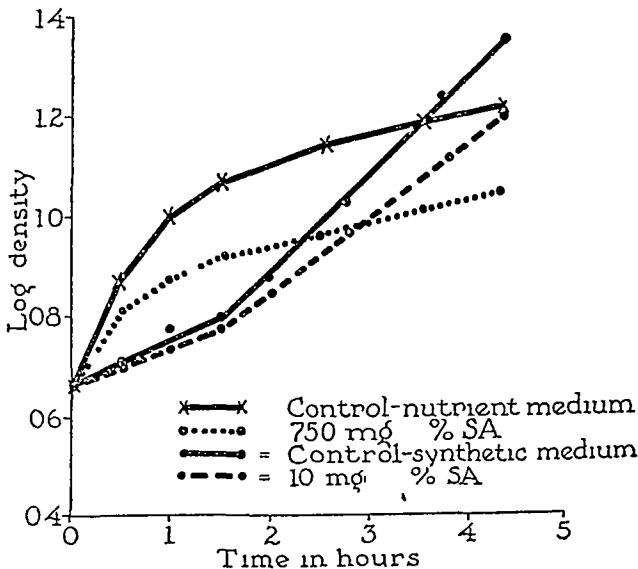


FIG. 1. THE EFFECT OF SULFANILAMIDE ON *E. COLI* I IN SYNTHETIC AND NUTRIENT MEDIA

the normal manner of growth of *E. coli* under the conditions of the experiment. In figure 1 are illustrated the typical growth curves of a smooth culture phase of *E. coli* I as determined by turbidimetric methods. Turbidimetric readings indicated that, in the synthetic medium, a lag phase of 1.5 hours occurred, followed by approximately a 3-hour logarithmic phase of growth, whereas in the nutrient broth no lag phase of growth could be detected by this method. The apparent absence of a lag phase in this medium was probably due to the immediate increase in cell mass. The viable plate counts gave similar results in both media, with first a short lag phase of 1 to 1½ hours, followed by the logarithmic phase of growth. The growth in the nutrient broth appeared to continue for 50 to 55 hours as compared to the 5- to 7-hour growth in the synthetic medium, and eventually reached a greater bacterial population than was found at the end of growth in the synthetic medium.

The effect of sulfanilamide on the growth of *E. coli* I when added with the inoculum. In figure 1 are shown also the growth curves as measured turbidimetrically of *E. coli* I in synthetic and in nutrient media with and without SA. In the presence of SA prompt partial inhibition of growth occurred. These results were confirmed by the plate counts,

The determinations of cell size (table 1) show that, in the cultures containing SA, cell size increased for the first 15 hours, followed by a decrease in length during the logarithmic phase of growth. These findings are in contrast to the control, in which length increased only for the first hour. SA in these experiments did not form large aberrant forms of *E. coli* I, as has been found by others with certain cocci (Boroff *et al.*, 1942).

Thus SA inhibited the growth of *E. coli* I promptly, and, since these results were unexpected owing to the large number of observations which report a delay in the bacteriostatic action of the sulfonamides, certain experimental conditions were altered in an attempt to explain this reaction.

(1) Use of larger and smaller concentrations of sulfanilamide with the same inoculum of *E. coli* I. Prompt inhibition of growth occurred regardless of the

TABLE 1

Cell length in microns of *E. coli* I at different times during growth in synthetic media

	TIME IN MINUTES							
	0	30	60	90	120	165	225	260
Without SA	2.75	2.85	3.8	3.18	2.68	2.37	2.23	2.09
With SA	2.59	3.09	3.47	3.54	2.35	3.08	2.44	2.345

concentration of SA employed in both nutrient and synthetic media. Concentrations of SA varying from 10 mg per cent to 40 mg per cent produced a proportionately greater inhibition of growth in the synthetic medium, and concentrations from 1 mg to 10 mg per cent of SA produced proportionately less inhibition. In the nutrient medium, owing to the insolubility of SA in concentrations greater than 750 mg per cent these were not used, however, less SA inhibited the growth proportionately less. These results confirmed those of other investigators that, for a given inoculum, inhibition was proportional to the concentration of the sulfonamide.

(2) The effect of increasing or decreasing the size of the inoculum on sulfonamide action. Different amounts of inoculum were used in both media with the standard concentrations of SA, and again it was found that prompt inhibition occurred in both media when SA was added immediately, but that the amount of inhibition varied. As the inoculum became larger, the inhibition of growth was less.

(3) The influence of the culture phase on the sulfanilamide action. The rough (R) culture phase of *E. coli* I was compared with the smooth (S) culture phase using the standard amount of inoculum and SA concentration, to determine

whether the culture phase influenced the SA action. It was found the R phase of *E. coli* was inhibited promptly in both media by SA. Moreover, there appeared to be no difference in sensitivity to SA between the two culture forms.

(4) The effect of sulfanilamide on another strain of *E. coli*. The second strain of *E. coli*, 6522, was used to determine whether the prompt inhibition of growth which occurred with the stock strain of *E. coli* was a characteristic of only that strain. The standard amount of inoculum of this organism was added to the media and to media containing the standard concentrations of SA, with the result that again prompt inhibition of growth occurred both in the synthetic medium and in nutrient broth. In the synthetic medium, the lag phase of growth with strain 6522 was shorter, and the generation time more rapid during the logarithmic phase than with *E. coli* I. Varying concentrations of SA added to the standard inoculum of strain 6522 organisms in the synthetic medium produced the same proportional inhibition as was obtained with *E. coli* I.

(5) Use of unwashed cells. Unwashed cells were used in place of the washed cells. However, prompt inhibition of bacterial growth by the SA occurred.

(6) Use of older bacterial cells. An inoculum from a 36-hour culture was used in the synthetic medium, and an inoculum from a 64-hour culture (after growth had stopped) was used in nutrient broth. Inocula from these cultures when added to medium containing the standard concentrations of SA again were inhibited promptly in both media. The effect of SA on cells younger than 18 hours will be discussed in the next section.

*The effect of SA on the growing cultures of E. coli.* (1) *Synthetic medium*. SA was added at 30-minute intervals to growing cultures of the smooth stock strain of *E. coli*. The inoculum was taken from an 18-hour culture. SA added during the first 1.5 hours, or during the lag phase of growth, produced prompt inhibition of bacterial growth. The amount of SA inhibition during the lag phase decreased with each later addition. When SA was added at 2 hours or at the time the logarithmic phase of growth was beginning, a delay in the SA inhibition occurred which lasted for 1 to 2 hours followed by the usual sulfanilamide inhibition. The results of a typical experiment are shown in table 2. SA was added also to the growing culture at 15-minute intervals with the same results.

The first set of plate counts was made of the control growth of *E. coli* and the growth to which SA was added at 1 hour. The plate counts confirmed the turbidity readings in that no delay was present. The second set of plate counts was made of the control growth of *E. coli* and the growth to which SA was added at 2 hours after inoculation, or at the beginning of the logarithmic phase of growth, and these results show a delay in the bacteriostatic action of SA of 1.5 hours.

The size of the bacterial cells was measured in the control culture and the culture to which SA was added at 2 hours, and it was found to vary in the same fashion in both cultures, therefore, there appeared to be no relationship between cell size and the delay in SA action.

The hydrogen ion concentration was measured during the growth of *E. coli*

in the synthetic medium for its possible effect on the delay in SA action. This is presented in table 3. At 2 hours, when a delay in the SA inhibition occurred, the pH of the medium was 7.0. This hydrogen ion concentration was not significantly lower than that of the medium initially.

TABLE 2

*The effect of adding 10 mg per cent SA to an E. coli I culture at various times during growth in synthetic medium*

TIME IN MINUTES	SYNTHETIC MEDIUM									
	Percentage of transmission of light									
	SA added with inoculum		SA added 1 hr		SA added 1½ hr		SA added 2 hr		SA added 2½ hr	
	C*	SA†	C	SA	C	SA	C	SA	C	SA
0	90 00	90 00	90 00	90 00	90 00	90 00	90 00	90 00	90 00	90 00
60	86 00	87 50	88 00	88 00†						
90	84 50	86 50	87 00	87 50	87 00	87 00†			83 50	83 50
120	78 25	84 75	82 00	86 00	82 00	84 50	80 00	80 00†	78 00	78 00
150	73 00	83 00	78 00	82 75	77 50	81 50	75 00	75 00	73 50	73 50†
180	66 50	77 00	71 25	77 00	71 00	76 50	68 50	68 50	69 00	69 00
210	60 00	73 00	65 00	72 50	63 50	71 00	63 00	63 00	63 00	63 00
240	56 00	70 00	60 00	69 00	59 00	68 00	57 50	59 00	56 75	57 50
270	52 00	66 75	54 50	65 50	54 00	64 00	52 50	55 00	52 75	53 75
300							50 50	54 00	49 50	51 00
375	50 50	56 00	50 50	55 00	49 00	53 00	50 50	53 00	48 00	49 50
18 hr later	49 00	51 00	49 00	50 00	49 00	49 00	49 00	49 00	48 00	48 00

\* Tube to which no SA was added

† Tube to which 10 mg per cent SA was added

TABLE 3

*The variation in the hydrogen ion concentration occurring during the growth cycle of E. coli I with and without SA*

SYNTHETIC MEDIUM	TIME IN MINUTES						
	0	60	120	180	240	300	330
pH of medium without SA	7 15	7 05	7 0	6 5	6 35	5 85	5 35
pH of medium with SA	7 17	7 1	6 9	6 9	6 9	6 75	6 7

In order to determine whether antagonists to SA action might be present in the medium and so interfere with the action of the SA to give a delay in its inhibition, two growing cultures of *E. coli* were centrifuged at 1 hour and at 2 hours after inoculation, and the supernatant was removed. To one tube of each set fresh medium was added, and to the other tube medium containing 10 mg per cent of SA. Both tubes were again incubated at 37.5 C. The culture to which SA was added at 1 hour was inhibited promptly by the SA, whereas the 2-hour

culture to which SA was added showed approximately a 1-hour delay in the SA action. These results (table 4) confirm the findings that (1) the delay in the SA action is not caused by a pH change, and (2) changes in the medium due to by

TABLE 4

*The effect of fresh synthetic medium added with the SA later in the E. coli growth cycle on the bacteriostatic action of SA*

TIME IN MINUTES	PERCENTAGE OF TRANSMISSION OF LIGHT					
	SA added with the inoculum		SA added after 1 hour growth		SA added after 2-hour growth	
	C*	SA†	C*	SA†	C*	SA†
0	90 00	90 00‡	90 00	90 00	90 00	90 00
60	86 50	88 00	86 50	85 75	86 00	86 00
90	82 50	87 75	Centrifuged			
120	78 00	85 00			77 00	78 00
150			82 00	82 00‡	Centrifuged	
195	64 50	79 00	76 00	79 00		
240	56 00	74 50	69 50	73 00	73 50	73 50‡
285	51 50	71 00	61 00	66 25	67 00	67 00
330	51 25	67 00	53 50	59 00	58 00	59 00
					48 75	51 25

\* Tubes to which no SA was added

† Tubes to which 10 mg per cent SA was added

‡ Time when the SA was added

TABLE 5

*The effect of 10.0 mg per cent SA in synthetic medium on larger inocula of E. coli I*

TIME IN MINUTES	PERCENTAGE OF TRANSMISSION OF LIGHT					
	78*		76		72	
	C†	SA‡	C†	SA‡	C†	SA‡
0	78 00	78 00	76 00	76 00	72 00	72 00
30			73 00	74 00		
60	67 50	71 50	69 00	71 25	59 00	64 50
90			63 00	65 00	51 00	57 25
105	60 00	65 00				
120			55 50	58 75	47 00	54 00
150	52 00	59 00	48 25	52 00		
180	49 50	53 00				
195					42 50	47 00
210			43 50	46 50		
225	49 50	51 75				

\* Size of inoculum as measured by turbidimetric reading

† Tubes to which no SA was added

‡ Tubes to which 10 mg per cent SA was added

products of bacterial growth do not influence or cause the delay in SA bacteriostasis.

As the cell population was 3 to 4 times greater at 2 hours or at the time of delay in the SA bacteriostatic action occurred, this larger number of cells was



used as an inoculum in the presence of 10 mg per cent of SA to determine its effect on the delay. However, since SA inhibited promptly the growth of this inoculum (table 5), the number of cells at the beginning of the logarithmic phase of growth was not important in producing the delay in the SA inhibition.

The last factor tested was the use of a larger concentration of SA, 20 mg per cent, and adding it at 30-minute intervals during bacterial growth. Again there was no delay in the SA action until logarithmic growth had begun. Thus, a larger concentration of SA did not affect the manner of SA inhibition.

TABLE 6

*The effect of adding 750 mg per cent SA to an E. coli I culture at various times during growth in nutrient broth*

TIME IN MINUTES	PERCENTAGE OF TRANSMISSION OF LIGHT									
	SA added with inoc.		SA added 1 hr		SA added 1½ hr		SA added 2 hr		SA added 2½ hr	
	C*	SA†	C	SA	C	SA	C	SA	C	SA
0	90 00	90 00	90 00	90 00	90 00	90 00	90 00	90 00	90 00	90 00
60	85 00	87 00	85 75	85 00						
90	82 50	86 00			83 00	82 00				
120			Centrifuged‡				79 50	80 00		
135			80 00	80 00†	Centrifuged					
150	79 00	83 50	71 00	75 00			Centrifuged		78 20	79 00
165					72 50	72 50†	77 00	77 00†	Centrifuged	
195	76 50	83 00	68 50	73 00	64 50	67 20			71 00	71 00†
225							67 00	67 00	62 50	62 50
255	75 00	82 25	66 50	72 00	62 00	63 75			62 00	62 00
270							66 00	65 70		
345	73 50	81 50	65 25	71 00	61 00	63 25	64 50	64 50	61 00	60 50
24 hr later	32 00	78 00	32 50	64 00	31 70	61 00	35 00	56 00	31 50	58 00

\* Tube to which no SA was added

† Tube to which 750 mg per cent SA was added

‡ Owing to the relative insolubility of SA, 750 mg just dissolved in 100 ml medium. Thus in order to add 750 mg per cent SA to a culture, it was first necessary to centrifuge the cultures, pour off supernatant medium, and then add either medium with or without SA.

The same results were obtained when 10 mg per cent of SA was added at 30-minute intervals during the growth of (1) *E. coli* 6522, (2) the rough culture phase of *E. coli* I, and (3) unwashed cells of *E. coli* I.

(2) *Nutrient medium* In table 6 are shown the results of adding 750 mg per cent of SA to *E. coli* I cultures at 30-minute intervals after inoculation. There was generally immediate inhibition of growth when SA was added for the first 1.5 hours. Although this experiment was repeated many times, the results did not check so regularly as those in which the synthetic medium was used. For example, when SA was added at 1 or at 1.5 hours after inoculation, there would be occasionally a delay in the bacteriostatic action of the SA. The explanation for these reactions might be based on the assumption that owing to the slower and more prolonged growth of *E. coli* in the nutrient medium, the cells used in the various inocula varied somewhat in their stage of growth. Thus in some

instances logarithmic growth occurred earlier, with the result that the delay in the SA inhibition also occurred earlier

Changes in cell size were found to be similar to those found in the synthetic medium, and in all other respects the results were identical with those obtained with the synthetic medium

#### DISCUSSION

It would appear from the results of this study that the presence or absence of a delay in the bacteriostatic action of sulfanilamide on *E. coli* is related to the age of the bacterial culture and to the physiological state of the bacteria at the time. No delay in the SA inhibition occurred when SA was added to an inoculum consisting of old cells, but one did occur when SA was added to cells which were present just before and during the logarithmic period of multiplication. Such factors as the pH of the medium, the number of organisms, the culture phase (R or S) of the organisms, or the presence of metabolic by-products in the medium were found not to influence this phenomenon.

These results also help to explain the discrepancies existing in the literature with regard to the absence or presence of the delay in the bacteriostatic action of SA on bacteria. Although many investigators (Strauss and Finland, 1941, Boroff *et al.*, 1942, Rose and Fox, 1942) do not state the age of the culture employed as an inoculum, the results obtained by a few that do (Lockwood, 1938, White and Parker, 1938, Kohn and Harris, 1941, Lowell *et al.*, 1941, MacLeod and Mirich, 1942) would indicate that the explanation might be the one that is brought out in the present study. These investigators used inocula from actively growing cultures and obtained a delay in the sulfonamide inhibition. Hobby *et al.* (1942) used an 18-hour culture of a streptococcus as an inoculum and obtained no delay in the sulfonamide action. There are, however, a few contrary reports. Libby (1940) used an actively growing culture of a pneumococcus as an inoculum and obtained no delay in the SA action. Muir *et al.* (1942) obtained a delay in the sulfonamide inhibition when using a 24-hour culture of *Salmonella enteritidis* as an inoculum. Sevag and Shelburne (1942) used an actively growing culture of *Streptococcus pyogenes* and obtained inhibition of bacterial respiration within 15 to 30 minutes. It should be pointed out that the chronological age of a culture is not necessarily related to the physiological age, as the latter will vary according to the nature of the bacterium and the environmental growth conditions.

Regardless of the mechanism involved, which can only be speculated upon, these findings, if found to apply to pathogenic bacteria, may have considerable significance from the standpoint of the prevention and treatment of infection with sulfonamides.

#### SUMMARY

Sulfanilamide when added to either synthetic or nutrient media together with an inoculum of *Escherichia coli* consisting of old cells produced prompt inhibition of growth.

This prompt inhibition shown by sulfanilamide on *E. coli* was not influenced by (a) the concentration of SA, (b) the size of the bacterial inoculum, (c) the strain of *E. coli* used, (d) the culture phase of the organism, or (e) the use of unwashed instead of washed cells for an inoculum.

A delay in the bacteriostatic action of sulfonamide occurred only when it was added to cultures of *E. coli*, in either synthetic or nutrient media, just prior to or during the logarithmic phase of growth.

Changes in the pH, the medium, or the bacterial population did not influence the occurrence of the delay in the bacteriostatic action.

Sulfanilamide did not significantly alter the size or shape of the individual cells in cultures of *E. coli*.

Young, actively metabolizing and multiplying cultures of *E. coli* are more resistant to the bacteriostatic action of sulfanilamide, whereas older and slowly metabolizing and multiplying cells of *E. coli* are more susceptible.

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# THE UTILIZATION OF NITROGEN IN HYDROXYLAMINE AND OXIMES BY *AZOTOBACTER VINELANDII*<sup>1</sup>

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Recent studies have provided several types of evidence supporting the view that ammonia is the key intermediate in nitrogen fixation by *Azotobacter*. An alternative hypothesis based on hydroxylamine depends almost entirely on the detection of traces of oximes in cultures fixing nitrogen. Although the two hypotheses are not mutually exclusive (Burris and Wilson, 1945), hydroxylamine remains an improbable intermediate unless it or its oximes can serve as a source of nitrogen for the organism. This important fact of utilization is disputed (Wilson and Burris, 1947), but quantitative experiments are few. To clarify this significant aspect of the mechanism of biological nitrogen fixation, we have re-examined the entire question in an effort to secure conclusive results.

## METHODS

Part of the confusion and controversy on this subject arises from the unsatisfactory methods for the estimation of oximes. We believe it desirable, therefore, to summarize not only our data on the reliability of the methods but also the exact procedures, two details missing from several important studies in this field.

**Hydroxylamine.** Endres' modification (Endres and Kaufmann, 1937) of Blom's method (1928) was used, in which hydroxylamine is oxidized to nitrite with iodine-acetate solution, with sulfanilic acid the nitrite forms a diazo compound that is coupled with  $\alpha$ -naphthylamine to form a red dye that is estimated colorimetrically. The reagents are:

**Sulfanilic acid.** 10.5 g sulfanilic acid, 6.8 g sodium acetate, 300 ml glacial acetic acid, and 600 ml distilled water. Boil 3 minutes and dilute to 1 liter.

**$\alpha$ -Naphthylamine.** To 1,000 ml of boiling distilled water add 5.0 g  $\alpha$ -naphthylamine and continue boiling for an additional 5 minutes. Filter hot and add enough hydrochloric acid (about 5 ml, concentrated) to clear of precipitate.

**Iodine-acetate.** 1.3 g iodine dissolved in 100 ml glacial acetic acid.

**Thiosulfate.** 2.5 g sodium thiosulfate dissolved in 100 ml distilled water. Keep in refrigerator. All reagents should be stored in brown bottles.

**Procedure.** To 1 to 5 ml of unknown solution containing about 1  $\mu$ g per ml hydroxylamine add 1 ml of sulfanilic acid reagent and 0.5 ml iodine-acetate, shake for 2 minutes, then decolorize excess iodine with thiosulfate and make to 8 ml with distilled water. Add 1 ml of  $\alpha$ -naphthylamine reagent, shake well, and read red color in photoelectric colorimeter at 500  $m\mu$ . The reliability of the

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method was demonstrated by preparing a standardization curve with purified sodium nitrite and estimating the hydroxylamine in samples of cp grade, recovery was quantitative. Because of certain variations tried when this method was used for oximes, the effect of altering the sequence of reagents was determined. If the excess iodine is removed before the addition of sulfanilic acid, no color is formed, if the solution of hydroxylamine is boiled with the sulfanilic acid before treatment with iodine-acetate, color is formed, but not if the heating is done in the presence of iodine-acetate.

*Oxime* Endres (1934) used the iodine-acetate solution both to hydrolyze oximes and to oxidize the resulting hydroxylamine, but we obtained color with the oximes of oxalacetic or  $\alpha$ -ketoglutaric acids only when they were hydrolyzed with sulfanilic acid. Four ml of a suitably diluted oxime solution were added to 4 ml of sulfanilic acid reagent, and the mixture was heated for 15 minutes in a boiling water bath. After cooling and making to volume, 1 to 2 ml were used

TABLE 1  
*Free and bound  $\text{NH}_2\text{OH}$  in oxime of  $\alpha$ -ketoglutaric acid*

0.2 M $\alpha$ -KETOGLOUTARIC ACID*	FREE $\text{NH}_2\text{OH}$		BOUND $\text{NH}_2\text{OH}$ †			CORRECTED RECOVERY‡
			Dilution	Total	Recovery	
ml	$\mu\text{g/ml}$	per cent		$\mu\text{g/ml}$	per cent	per cent
1	1.58§	23.8	1:20	1.78	53.9	39.5
2	0.50	0.8	1:20	1.13	34.2	33.8
3	0.55	0.8	—	—	—	—
5	0.75	1.1	1:10	0.65	9.8	8.8

\* Added to 1 ml 0.2 M  $\text{NH}_2\text{OH}$  (66  $\mu\text{g}$ ) and kept 24 hours at room temperature

† Total  $\text{NH}_2\text{OH}$  after hydrolysis with sulfanilic acid

‡ Bound  $\text{NH}_2\text{OH}$  corrected for free  $\text{NH}_2\text{OH}$  found

§ 1:10 dilution

for the analysis of free hydroxylamine. When a preparation of acetoxime (Eastman Kodak Company) was dissolved, 22 per cent of the calculated hydroxylamine was found by the Blom-Endres method, after hydrolysis with sulfanilic acid, an additional 70 per cent was obtained. If, however, the iodine-acetate method of hydrolysis was used, an increase of only 4.5 per cent was observed. The recovery by this method was less satisfactory with the oxime of  $\alpha$ -ketoglutaric acid, as can be seen by the data in table 1. Endres stated that this method estimates only one-sixth of the nitrogen in oximes, but he apparently believes it is nevertheless reliable as he uses data obtained with it for quantitative conclusions. Our experience suggests that, although under carefully standardized conditions recoveries greater than 16 per cent are possible and results less erratic than those in table 1 can be obtained, the method is at best only semiquantitative.

In general, estimation of oxime nitrogen by the Kjeldahl method is unsatisfactory, the oximes of pyruvic, oxalacetic, and  $\alpha$ -ketoglutaric acids are no ex-



ceptions, as shown in table 2. In an attempt to reduce losses from the presence of free hydroxylamine, three equivalents of the keto acids were used. Although this reduced the free hydroxylamine to 1 per cent or less of the total nitrogen, recoveries with pyruvic and oxalacetic acids were still low. In cultures, however, the recovery with these appeared to be higher, 75 to 85 per cent. Although the microkjeldahl method used (Wilson and Knight, 1947) is unsatisfactory for exact results, it appeared to be superior to Endres' method and was therefore used as a roughly quantitative measure of oxime.

TABLE 2  
*Recovery of oxime-N by microkjeldahl method*

OXIME OF	ORIGINAL N	DILUTION	N FOUND	RECOVERY	FREE NH <sub>2</sub> OH
	<i>μg/ml</i>		<i>μg/ml</i>	<i>per cent</i>	<i>per cent</i>
α-Ketoglutaric acid	2,000	1 125	17 75	104 7	0 24
		1 100	20 0	100 0	0 28
		1 125	15 5	96 7	0 28
	750	1 25	27 5	91 7	0 43
			28 0	93 3	0 38
	500	1 50	10 0	100 0	0 17
		1 31 25	15 5	96 8	0 36
		1 55 5	7 5	83 3	0 10
Oxalacetic acid	750	1 25	21 5	74 1	0 43
	500	1 62 5	5 0	62 5	0 04
			5 5	68 8	0 23
			4 0	50 0	0 01
			5 0	62 5	0 07
Pyruvic acid	750	1 25	17 0	56 6	0 46
			16 5	55 8	0 39
	500	1 50	3 5	35 0	1 10
		1 31 25	6 5	40 6	0 50

3 X keto acid equivalent added to NH<sub>2</sub>OH HCl brought to neutrality

*Preparation of the oximes* Because of the toxicity of free hydroxylamine for *Azotobacter* it is essential that the dissociation of the oxime be minimized. As can be seen in table 1 this can be done by adding excess keto acid, for example two to three equivalents of the hydroxylamine to be neutralized. A neutral reaction also favors the formation of oxime. The calcium salt of α-ketoglutaric acid<sup>2</sup> was decomposed with sodium carbonate since the usual procedure of pre-

<sup>2</sup> We thank the Fermentation Division, North Regional Research Laboratory, USDA, Peoria, Illinois, for generously supplying us with samples of both crude calcium ketoglutarate and pure α-ketoglutaric acid.

precipitating the calcium with oxalic acid resulted in a toxic preparation. Oxalacetic acid was prepared by the method of Krampitz and Werkman (1941), both pyruvic acid and sodium pyruvate (Eastman Kodak Company) were used for making the oxime of pyruvic acid.

*Bacteriological methods* The medium was Burk's nitrogen-free salts plus 2 per cent sucrose (Wilson and Knight, 1947). *Azotobacter vinelandii*, strain *Original*, was cultured in this medium by transfer every 4 days. The purity of culture was checked by microscopic examination (gram stain) and by inoculation of beef extract peptone medium, in which *Azotobacter* grows slowly and contaminants are accordingly favored.

#### EXPERIMENTAL PROCEDURES

To determine the toxicity of hydroxylamine, 1 ml of solution containing 20 to 200 micrograms was added to 20 ml of the nitrogen-free medium, four bottles of each concentration were prepared, two of which were inoculated with a loop of a 24-hr culture of *Azotobacter vinelandii*.

In the media containing hydroxylamine, no growth was evident for 48 to 72 hours after which the growth apparently was normal. Quantitative estimation of hydroxylamine in the uninoculated controls demonstrated that this compound disappeared rapidly, so that by 24 hours the concentration was less than 1  $\mu\text{g}$  per ml, even in those originally containing as much as 10  $\mu\text{g}$  per ml. Apparently, growth was delayed until the concentration of hydroxylamine was definitely less than 1  $\mu\text{g}$  per ml, a figure in agreement with Burk's rough estimate of 0 to 3 mg per L (Burk and Horner, 1935). Hydroxylamine disappeared from the medium when it was sterilized by filtration through a Seitz as well as in the autoclave. When kept in a refrigerator, the disappearance was slower but not prevented. The instability of hydroxylamine in the medium prevented critical test by our technique of its utilization by *Azotobacter* in a nontoxic concentration (less than 1  $\mu\text{g}$  per ml). From experiments with the more sensitive microrespiration method, Burk and Horner (1935) concluded that it is not metabolized.

In the trials with oximes, an excess of the keto acid was always used to reduce the free hydroxylamine below a toxic concentration. An experiment with the oximes consisted of the following duplicate bottles (20-ml) inoculated with 0.1 ml of a 24-hr culture of *Azotobacter vinelandii*.

#### I Atmosphere of 80 per cent hydrogen and 20 per cent oxygen

- 1 Experimental plus 0.2, 0.5, 1.0, or 2.0 mg per 20 ml of oxime-N
- 2 Inorganic-N control plus same concentrations of combined nitrogen as ammonium nitrate or sulfate
- 3 Control on atmosphere no source of combined nitrogen
- 4 Control on toxicity of oxime plus 2.0 mg oxime-N and 2 mg inorganic N per 20 ml

#### II Air controls

- 1 Control on fixation of nitrogen no sources of combined N
- 2 Inorganic-N control plus 2 mg inorganic N per 20 ml

In each of two desiccators a bottle of each duplicate of the hydrogen oxygen

series was placed. The desiccators were evacuated to about 10 inches of mercury, and filled with hydrogen, this was repeated 5 times, then filled with an atmosphere of 80 per cent hydrogen and 20 per cent oxygen. A manometer was attached and a slight vacuum was drawn to check for leaks during the experi-

TABLE 3  
*Growth of Azotobacter vinelandii on oxime of oxalacetic acid*

N PER BOTTLE (20 ML)	ATMOSPHERE	OXIME SERIES*		NH <sub>4</sub> NO <sub>3</sub> SERIES	
		Free NH <sub>2</sub> OH	Total N†	Turbidity	N in cells
Experiment I					
micrograms		μg/ml	micrograms		micrograms
0	H <sub>2</sub> O <sub>2</sub>			100 0	—
				100 0	—
200		0 00	—	73 5	150
		0 00	—	68 0	122
500		0 05	—	51 0	345
		0 20	—	54 5	328
1,000		0 10	910	28 0	650
		0 00	950	28 0	675
2,000		0 08	1,675	12 0	1,450
		0 10	1,700	10 5	1,375
4,000‡	Air			18 0	813
				11 5	1,287
2,000				3 0	3,875
				3 0	3,925
0§				4 0	2,875
				4 5	3,250
Experiment II					
0	H <sub>2</sub> -O <sub>2</sub>			100 0	—
200		0 00	—	74 0	128
500		0 15	—	46 2	325
1,000		0 19	912	23 2	700
2,000		0 29	1,712	10 5	1,625
4,000‡	Air			20 5	963
2,000				4 2	3,375
0§				3 8	3,488

\* Turbidity in cultures of oxime series same as 0 control, readings measure light transmitted

† Recovery at end of the experiment

‡ 2,000 μg NH<sub>4</sub>NO<sub>3</sub>-N plus 2,000 μg oxime N—toxicity control

§ Fixation control

ment. Cultures were incubated for 60 hours at 30 C together with the air controls, at harvest, the turbidity was estimated in a Coleman photoelectric colorimeter (530 mμ), after which the cells were removed by centrifugation. Cellular nitrogen and free hydroxylamine were determined, oxime-N was also estimated in the two highest concentrations used.

## RESULTS AND DISCUSSION

Details of two experiments with the oxime of oxalacetic acid are given in table 3, the data of each sample are supplied for experiment I, the mean of the duplicates for experiment II. Similar results with the oximes of  $\alpha$ -ketoglutaric acid and pyruvic acid are summarized in table 4. In the hydrogen-oxygen series there was no evidence of growth on oxime-N, growth in the inorganic-N controls when measured by either turbidity or cellular nitrogen was proportional to the quantity of nitrogen supplied. The lack of growth in the media containing oxime-N arises from inability to use this type of combined nitrogen rather than from toxicity of the free or bound hydroxylamine since (a) estimation of free

TABLE 4

*Test of growth of Azotobacter vinelandii on oximes of  $\alpha$ -ketoglutaric and pyruvic acid*

NITROGEN PER BOTILE (20 ML)	$\alpha$ KETOGLUTARIC ACID*			PYRUVIC ACID*		
	Free $\text{NH}_2\text{OH}$ in oxime	$(\text{NH}_4)_2\text{SO}_4$ control		Free $\text{NH}_2\text{OH}$ in oxime	$\text{NH}_4\text{NO}_3$ control	
		Turbidity	N in cells		Turbidity	N in cells
micrograms	$\mu\text{g/ml}$		micrograms	$\mu\text{g/ml}$		micrograms
0	—	100 0	—	—	100 0	—
200	0 25	81 2	125	0 20	66 1	125
500	0 49	59 2	312	0 38	38 0	294
1,000	0 98	37 5	694	0 70	19 2	644
2,000	1 19	22 5	875	1 20	9 8	1,388
4,000†		24 2	924		10 7	1,175
2,000‡		21 0	775		3 2	3,725
0§		3 2	3,350		3 7	3,012

Recovery of oxime-N was 80 to 85 per cent in  $\alpha$ -ketoglutaric series, 70 to 75 per cent in pyruvic acid series

\* No turbidity in oxime series in  $\text{H}_2\text{-O}_2$

† 2,000  $\mu\text{g}$  inorganic nitrogen plus 2,000  $\mu\text{g}$  oxime nitrogen

‡ Inorganic nitrogen control in air

§ Fixation control grown in air, all others grown in 80 per cent hydrogen, 20 per cent oxygen mixture

hydroxylamine indicated nontoxic levels, (b) growth on inorganic-N was only slightly depressed if the highest concentration of oxime-N used was also supplied, and (c) on transfer to air the cultures fixed nitrogen in the presence of the oxime-N (table 5)

Other points of interest brought out by the data in tables 3, 4, and 5, include (a) recovery of oxime-N was complete within the large experimental error, (b) the highest concentration of oxime-N (100  $\mu\text{g}$  per ml) was slightly toxic, probably because of free hydroxylamine, as is evident by the somewhat smaller uptake of inorganic nitrogen in its presence, (c) the limiting factor for growth on  $\text{NH}_4\text{NO}_3$  in hydrogen-oxygen is the supply of assimilable N, as is shown by the superior growth in air in which fixation can take place as soon as the combined nitrogen has disappeared, (d) if ammonium sulfate is the source of inorganic

nitrogen, lowering of the pH by the residual sulfate radical is the limiting factor for growth, and (e) the lack of quantitative recovery of inorganic nitrogen in the cells of the hydrogen-oxygen series and the apparent more than quantitative recovery of added oxime-N when cultures were transferred to the air (table 5) probably is caused by the soluble nitrogen liberated from the bacteria.

The results in the tables are typical of many replicate experiments completed five were made with the oxime of  $\alpha$ -ketoglutaric acid using both crude and pure samples of the keto acid, three were made with the oxime of oxalacetic acid, and two were made with the oxime of pyruvic acid. In a hydrogen-oxygen atmosphere, *Azotobacter* failed to grow on oxime-N in any of the 10 trials.

TABLE 5

*Fixation of molecular nitrogen by Azotobacter vinelandii in presence of oximes*

OXIME N PER BOTTLE (20 ML.)	$\alpha$ KETOGLUTARIC ACID SERIES		OXALACETIC ACID SERIES	
	N in cells	N in medium	N in cells	N in medium
	micrograms	micrograms	micrograms	micrograms
200	2,300	550	1,650	—
	1,200	400	2,450	656
500	375	612	1,550	687
	1,200	—	2,425	459
1,000	2,525	1,325	1,375	1,200
	1,200	—	—	1,300
2,000	2,000	1,625	1,575	2,250
	1,000	2,000	1,900	2,250

Cultures incubated for 60 hours in  $H_2-O_2$  mixture, no visible turbidity, then transferred to air for 72 hours

We have repeatedly emphasized that one property of a proposed intermediate is that it should immediately and completely supplant fixation (Wilson and Burris, 1947), a point of view analogous to the recent proposal of Stanier (1947) for the test of proposed intermediates. We have demonstrated that of the various sources of inorganic and organic nitrogen used by *Azotobacter*, ammonia (and urea) alone possesses these two properties. Except for ammonia (urea), even when a compound does compete with the nitrogen-fixing reaction, a period of adaptation is required if the organism has been previously cultured on free  $N_2$  (Lind and Wilson, 1942, Wilson, Hull, and Burris, 1943, Burris and Wilson, 1946). This criterion of "simultaneous adaptation," as Stanier calls it, cannot be applied to the likely intermediates of the "oxime" hypothesis because they fail to meet the even more necessary test of utilization. The possibility remains, of course, that difficulties of permeability account for this, but until positive evidence in support of this view is provided, rejection of the hydroxylamine and the accompanying oxime hypotheses appears logical.

## SUMMARY

In spite of unsatisfactory methods for the estimation of combined hydroxylamine, quantitative experiments on the utilization of both free and combined forms of this source of nitrogen can be made

Free hydroxylamine disappears rapidly from sterile Burk's N-free medium. The compound is extremely toxic to *Azotobacter*, and its utilization in non-toxic concentrations (less than 1  $\mu$ g per ml) is doubtful.

A series of carefully controlled trials demonstrated that the nitrogen in the oximes of pyruvic, oxalacetic, and  $\alpha$ -ketoglutaric acids is not available to *Azotobacter vinelandii*. This lack of utilization prevents the use of the specific critical tests that previously established ammonia as an intermediate in nitrogen fixation by this organism.

Until utilization of oxime-N is demonstrated so that the critical test can be applied, the hydroxylamine and oxime hypotheses should be rejected.

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# CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

## V COMPARATIVE EFFECTS OF RIBONUCLEASE, COBRA VENOM, AND PENICILLIN ON SUSCEPTIBLE BACTERIA<sup>1</sup>

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The present work is a continuation of our studies of the changes induced by penicillin and certain other diffusible substances in susceptible test organisms. Proper application of suitable staining techniques to assay plates clearly demonstrates inhibition zones around "penicylinders" containing bacteriostatic concentrations of penicillin within 2 to 3 hours after the antibiotic has begun to diffuse through a test plate, although no measurable zones are evident on untreated plates until several hours later. Among the reagents that have been useful for revealing zones of inhibited bacterial activity are appropriate redox indicators, notable among which is triphenyl-tetrazolium chloride,<sup>3</sup> and certain dyes that have been recommended for the detection of nucleic acid. A summary correlating our work with other published pertinent observations bearing on the mechanism of penicillin action is now in press (Pratt and Dufrenoy, 1948).

The purpose of the present paper is to report similar experiments with reagents that can be used to discriminate between ribonucleic and desoxyribonucleic acid derivatives. Ribonucleic acid derivatives, which largely constitute the "basophilic" material in bacteria (Sarciron, Vendrely, and Briand, 1945, Vendrely and Lipardy, 1946), preferentially adsorb pyronine from the Pappenheim mixture and are specifically amenable to enzymatic digestion by ribonuclease. They may be considered to represent the bulk of the "cytoplasmic" part of the test organisms in contrast with the "nuclear" desoxyribose nucleic derivatives (Sarciron, Vendrely, and Briand, 1945, Henry, Stacey, and Teece, 1945, Vendrely and Lipardy, 1946, Stacey, 1947, Boivin, Vendrely, and Tulasne, 1947). The latter preferentially adsorb methyl green from the Pappenheim mixture and are resistant to the action of ribonuclease. The ribo- and desoxyribonucleic acid derivatives may be differentiated also by their response to the Feulgen stain. However, we have found for bacteria as Carr (1945) concluded from his studies of tissues that "for the demonstration of these adsorption differences the use of acidified fuchsin is more convenient than the complications of the Feulgen reaction using the decolorized fuchsin."<sup>4</sup> Ribose nucleic acid derivatives contribute to

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<sup>4</sup> The comparative merits of the two methods have been discussed by Stowell (1946).

the "basophily" of the test organisms, since they adsorb dyes such as methylene blue or basic fuchsin more strongly as the pH is raised in the range from 3 to 7 (Dempsey and Singer, 1946)

#### EXPERIMENTS AND RESULTS

The procedures used in the present experiments were essentially as previously described (Dufrenoy and Pratt, 1947*a,b*, Pratt and Dufrenoy, 1947*a,b,c*). The work reported here is from assay plates seeded and preincubated for 2 to 3 hours in order to permit the organisms to reach the logarithmic phase of growth. Then the plates were "cylindred" and penicillin or any other agent under test was permitted to diffuse during a second incubation period of 3 hours in the case of *Staphylococcus aureus*, or 2 hours in the case of *Bacillus subtilis* or *Proteus vulgaris*. After the second incubation period the latent images of the inhibition zones were revealed by carefully flooding the plates with appropriate solutions. Patterns revealed by reagents intended to discriminate between ribo- and deoxyribonucleic acid derivatives matched those revealed by the redox indicators discussed in previous papers or by solutions of triphenyl-tetrazolium chloride.

*Test organisms exposed to penicillin alone* When penicillin assay plates are flooded for a few seconds with the Pappenheim mixture of methyl green and pyronine, then are rinsed with distilled water, differentiated with alcohol, and rinsed with methylal, the inhibition zones appear as blue green throughout, except for a narrow colorless rim that outlines each zone. The circumjacent ring of enhanced growth is clearly contrasted by its purple-red color against the inhibition zone on one side and the uninhibited background on the other side. The pattern obtained results from two phenomena, namely, differential adsorption and oxidation reduction. The oxidation reduction has been discussed previously, and the present technique confirms our published results to the effect that outside the zone of inhibition the test organisms rapidly reduce appropriate dyes to the leuco bases, whereas they lose that reducing property under the influence of penicillin or other bacteriostatic agents. The differential adsorption, which the Pappenheim mixture demonstrates by virtue of the preferential red staining of ribonucleic acid derivatives by pyronine, and blue-green staining of desoxyribonucleic acid derivatives by methyl green, results in red coloration in the ring of enhanced growth, which is crowded with actively growing test organisms that are rich in "basophilic" substances.

*Test organisms exposed to ribonuclease* According to Brachet (1941), pyronine stains specifically the pentosenucleic acid derivatives (the so-called basophilic materials). He reported that ribonuclease digests these compounds away and strips off the pyronine-staining cellular constituents. This can be verified on assay plates. Plates were prepared and preincubated in the usual way. Then solutions of penicillin or of ribonuclease or of penicillin plus ribonuclease were placed in the cylinders, and the plates were reincubated.

Solutions of ribonuclease alone in all concentrations used (1 to 50  $\mu\text{g}$  per ml) produced zones which could be revealed after 3 hours by the Prussian blue technique (0.5 per cent aqueous solution of potassium ferricyanide followed by 0.5



per cent aqueous solution of ferric sulfate) or other appropriate techniques previously reported for "developing" penicillin assay plates. Treatment of plates exposed to ribonuclease solution with Pappenheim's stain revealed blue-green inhibition zones outlined by reddish rings. This confirms for microorganisms the conclusion reached by Brachet (1941) on tissues, that ribonuclease strips away the ribonucleic acid compounds, leaving behind the desoxyribonucleic residues, which stain with methyl green.

Similar results were observed when cobra venom was substituted for ribonuclease in the cylinders. This result was not unexpected, since cobra venom has been shown recently to be capable of hydrolyzing a solution of pentose complexes (Pallares, Orozco, and Carvallo, 1947).

*Test organisms exposed to cobra venom.* Our interest in comparing the effects of cobra venom with those of penicillin was awakened by the reports of Rousseau and Pascal (1938a,b) that cells of streptococci exposed to constituents of cobra or viper venoms undergo swelling, and ultimately lysis, and that in such cells the basophilic materials (i.e., pentose- or ribonucleic acid derivatives) are stripped from the cells in 3 hours, leaving behind the "lyso-resistant nuclei," i.e., the desoxyribonucleic acid derivatives. Our interest was stimulated further by their interpreting their observations in terms of a shift of the equilibrium between S—S and —SH groups. Their interpretation was soon confirmed by other workers who found that cobra venom mediates a shift of the equilibrium of S—S and —SH groups (Binet, Weller, and Robillard, 1939).

Cobra venom in normal saline (10 mouse units per ml) and a mixture of cobra venom (5 mouse units per ml) and penicillin (0.5 units per ml) in phosphate buffer at pH 6.9 were permitted to diffuse for 3 hours from "penicylinders" on preincubated plates of *S. aureus*. Then the plates were treated with the Prussian blue reagent, whereupon typical very faintly bluish inhibition zones, each surrounded by an intensely blue ring of enhanced growth, appeared. The average diameter of the zones produced by penicillin plus cobra venom was 17 mm, the diameter of zones around cylinders containing the venom alone was 12 mm. Penicillin alone (0.5 units per ml) produced zones that averaged 13.5 mm.

In all cases microscopical examination of the plates revealed the same sequence of events that has been observed consistently with penicillin. In the inhibition zones, the original colonies, which had developed during the primary incubation period, failed to increase during the secondary incubation, and the organisms lost the ability to reduce ferric to ferrocyanide, thus accounting for the lack of pronounced staining in this area. The margin of each zone was outlined by a ring of enhanced growth where the original colonies had enlarged markedly.

#### DISCUSSION

It is well known that the response of test organisms to penicillin depends in large measure upon their physiological state, the organisms being most susceptible to the bacteriostatic or lytic action when they are growing most rapidly. Thus to secure the most striking response on assay plates treated with the various reagents that we have used it is necessary to permit the organisms to reach

the logarithmic phase of growth, during which they develop intense dehydrogenase activity before they fall under the influence of the antibiotic or lytic agents

On assay plates that have been "preincubated" to permit the organisms to reach the period of logarithmic growth before application of the substance under test, solutions of penicillin above 0.5 units per ml concentration, of ribonuclease above 1  $\mu\text{g}$  per ml,<sup>5</sup> or of cobra venom above 10 mouse units per ml<sup>5</sup> diffusing from "penicylinders," produce in 2 to 3 hours of secondary incubation zones of inhibition which, though not directly evident, may be clearly revealed by gently flooding the plates with appropriate reagents such as were used by Goyan, Dufrenoy, Strait, and Pratt (1947), Dufrenoy and Pratt (1947a,b), and Pratt and Dufrenoy (1947a,b,c). On penicillin assay plates so treated, log concentration of penicillin is a linear function of log diameter of inhibition zone over the range of concentrations from 1 to 8 units per ml. On conventional 16 hour assay plates the zone corresponding to a given unitage of penicillin is larger, but there is a break in the log regression line, the relationship between log concentration and log diameter no longer being linear over the entire range. The rectilinear log-dosage log-response relation also holds for solutions of ribonuclease on "three-hour" assay plates over the range 1 to 50  $\mu\text{g}$  per ml, but the slope of the curve (0.028) is much less than for penicillin (0.11). As the time for diffusion of ribonuclease was lengthened to 16 hours, there was but little increase in the size of the inhibition zone corresponding to a given concentration of enzyme. These results were to be expected, since Loring, Carpenter, and Roll (1947) showed that, when yeast ribonucleic acid was hydrolyzed by ribonuclease, there was at first a rapid rate of hydrolysis for about 5 hours, which was followed by a slower, relatively constant rate that was not affected by further addition of enzyme. The remaining fraction was relatively resistant to hydrolysis.

It has been shown that cobra venom as it diffuses through seeded plates induces cytochemical changes which can be interpreted as evidence of hydrolysis of pentose complexes (ribonucleic as contrasted to the desoxyribonucleic acids) and that these changes are similar to those produced by penicillin or by ribonuclease in the test organisms. In addition, it is known that cobra venom is endowed with acylase activity (Bovet-Nitti, 1947) and that it interferes with —SH groups on which dehydrogenase activity may depend.

The dehydrogenases involve ribonucleic acid in the basophilic material (staining red with pyronine) and active —SH groups, which we believe are responsible for the Prussian blue reaction in our tests. The specificity of the reaction was demonstrated by adapting to our work the technique of Genevois (1947), which calls for blocking of the —SH group by treatment with 10 per cent aqueous solution of sodium bromacetate at pH 7.5 for 10 minutes at 40°C. Experimentally, applying spot tests to the margins of inhibition zones, we found that are is of the plates so treated failed to give a positive Prussian blue reaction, whereas untreated areas gave the usual response in the ring of enhanced growth outlining the zones.

<sup>5</sup> Lower concentrations were not studied except in combination with low concentrations of penicillin.

The involvement of dehydrogenases in the responses obtained on assay plates may be further demonstrated by the use of triphenyl-tetrazolium chloride, which, according to Mattson, Jensen, and Dutcher (1947), is able "to act as an electron acceptor for many pyridine nucleotide dehydrogenases" When an uncolored aqueous solution of triphenyl-tetrazolium chloride (0.5 per cent) is gently flooded on the plates, the ring of enhanced growth immediately becomes the site of reduction of the compound to the red, insoluble formazan<sup>6</sup>

#### SUMMARY

Agar plates seeded with *Staphylococcus aureus*, *Bacillus subtilis*, or *Proteus vulgaris* were incubated to permit the organisms to reach the logarithmic phase of growth During this primary incubation period of two hours in the case of *B. subtilis* and *P. vulgaris*, or three hours in the case of *S. aureus*, the organisms developed high dehydrogenase activity Then aqueous solutions of penicillin, of ribonuclease, of cobra venom, or of mixtures of penicillin with ribonuclease or cobra venom, were placed in "penicylinders" on the plates and were permitted to diffuse through the agar for two to three hours during a second period of incubation

At the end of this time, inhibition zones could be clearly revealed by flooding the plates with appropriate reagents, although no such zones were evident on untreated plates When Pappenheim's stain, which may be used to discriminate between ribo- and desoxyribonucleic acid derivatives, was applied to the plates, the inhibition zones were seen to be areas in which the organisms had been stripped of their basophilic (ribonucleic acid) constituents, while each zone was surrounded by a ring in which the organisms exhibited marked basophilia

Application of solutions of redox indicators such as triphenyl-tetrazolium chloride, which may be used as sensitive tests for dehydrogenase activity, revealed very low, if any, dehydrogenase activity within the zones of inhibition, but each zone was surrounded by a ring of intense reducing activity, which corresponded exactly with the intensely basophilic areas revealed by Pappenheim's stain The results were the same whether the diffusing compound on the test plates was penicillin, ribonuclease, or cobra venom The possible significance of these observations in elucidating the cytochemical mechanism of penicillin action is discussed

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# A SIMPLE MEDIUM FOR IDENTIFICATION AND MAINTENANCE OF THE GONOCOCCUS AND OTHER BACTERIA

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The media generally used for the cultivation of the gonococcus have certain disadvantages. Most of them are unable to maintain freshly isolated cultures in a viable state for more than a few days. They may require ingredients likely to be variable in quality (e.g., meat infusion) or not readily available (e.g., horse serum). Moreover, the incorporation of such materials as ascitic fluid (Carpenter, 1945; Mahoney, Van Slyke, Cutler, and Blum, 1946) or serum (Peizer, 1942) in fermentation media requires special procedures for sterilization or aseptic handling that may be difficult to manage in the small laboratory.

The medium here described has none of these drawbacks. It is composed of available and relatively stable materials, can be sterilized in the autoclave, and supports continued growth of freshly isolated gonococci if transferred at intervals of 10 days, or even longer for many of the strains tested. In addition, it is a reliable base medium for the performance of fermentation tests. Its chief constituent is pancreatic casein digest, which has been shown (Vera, 1944) to be useful in the identification of clostridia.

## EXPERIMENTAL METHODS AND RESULTS

*Pancreatic digest of casein.* This was prepared by a plant-scale adaptation of the method of Leifson (1943) to meet the specifications set forth by the National Institute of Health in 1945 and now included in the United States Pharmacopoeia XIII. Of the qualifications specified, two characteristics were regarded as particularly pertinent in the present connection, namely, suitability for hydrogen sulfide production and freedom from fermentable carbohydrates. In preliminary studies with broths made from such digests of casein, several typical strains of typhoid bacilli and *Salmonella schottmuelleri* produced little or no blackening of lead acetate paper in 24 hours, and *Salmonella paratyphi* and *Shigella paradysenteriae* caused no darkening in 48 hours. In contrast, all of the test organisms readily gave positive reactions for hydrogen sulfide when they were grown in broths containing meat peptone or meat infusion. The result of this biological test is in harmony with the report of Block and Bolling (1945) that casein is deficient in available sulfur compounds, especially cystine.

In respect to fermentable carbohydrate, tests with cultures of *Escherichia*, *Aerobacter*, *Salmonella*, *Clostridium*, and cocci showed no acid production in 48 hours in casein digest broth, but showed definite acid production in broths containing meat infusion or meat peptone.

The use of cystine to supplement the digest of casein, as suggested by Boor

(1942), improved the properties of the substrate as a culture medium, but the results were not uniform. It was found, however, that the addition of both cystine and sodium sulfite provided the necessary conditions for good growth of freshly isolated as well as stock cultures of gonococci and other bacteria.

*Supplemented casein digest agar* The basic semisolid medium used in the investigation had the following composition in grams per liter of distilled water

Pancreatic digest of casein	20.0
Cystine	0.5
Sodium sulfite	0.5
Sodium chloride	5.0
Agar	3.5
Phenol red	0.017

All chemicals were cp grade and the agar was of the highest bacteriologically tested quality. The medium was prepared with and without added carbohydrate (0.5 per cent or, rarely, 1.0 per cent) and, after adjustment for a final pH of 7.3, was tubed and autoclaved at 116 to 118 C for 15 minutes. The sterile tubes were stored at room temperature, and used as needed.

Inocula of organisms suspected of being gonococci or meningococci were spread over the surface of the medium, other inoculations were made by stabbing. Cultures of *Neisseria* and of *Brucella abortus* were incubated in candle jars to supply an atmosphere enriched with carbon dioxide, unless otherwise stated.

The culture tubes were plugged with cotton. Although it was considered undesirable to disregard precautions against desiccation of the media, sterile or inoculated, it was not feasible under the experimental conditions to stopper the tubes as recommended by Carpenter and Shepard. Nevertheless, the results obtained clearly indicate the practical utility of the medium.

*Fermentation studies with Neisseria spp* Thirty-five strains of gonococci were transferred from chocolate agar slants, which were primary subcultures from diagnostic plates, to the basic medium and to the basic medium with glucose or maltose added. All of the cultures in the glucose medium developed an acid reaction within 24 hours. The plain and maltose cultures showed an alkaline change of the indicator at that time. This alkaline shift has been found to be characteristic of all strains thus far examined.

Transfers were made directly from 172 colonies on chocolate agar diagnostic plates<sup>1</sup> into maltose medium, 16 to 20 hours later, growth and alkalinity were visible in 150 tubes, and within 48 hours an additional 17 strains had grown. During the course of the experiments an occasional transfer failed to grow, but cultures were obtained from all plates having oxidase-positive colonies of gram-negative cocci. Glucose was fermented by all of the strains.

A series of 58 colonies from diagnostic plates was transferred into the casein digest medium with and without cystine and sulfite. All of the strains grew in

<sup>1</sup> The plates were obtained from the Bureau of Laboratories of the Baltimore City Health Department, through the courtesy of T. C. Buck, Jr., Assistant Director.

the presence of the sulfur compounds, but only 20 developed in their absence. Of 30 stock cultures also studied, 12 were able to grow in the absence of the sulfur compounds.

Twenty-eight strains of meningococci produced typical fermentation reactions in 16 to 24 hours in the supplemented casein digest medium in the presence of added carbohydrates. When tested in the medium without cystine and sulfite, the cocci grew, though more slowly, thereby delaying the appearance of a color change of the phenol red.

*Maintenance of Neisseria* In the absence of fermentable carbohydrate the gonococci remain viable for prolonged periods in this medium. By transfer at 10- to 14-day intervals, 251 strains have been maintained for at least 3 months, and some of them for over 3 years, 155 of the group were stored at 37 C, and the remainder were held at room temperature. Young cultures of gonococci from all strains, including those maintained as long as 3 years on this medium, showed the cellular morphology typical of the species.

Transplants from a limited number of cultures made even 3, 4, or 5 weeks after inoculation usually grew out, although after such long storage the original cultures had darkened and dried considerably. The poorest results were obtained from a series of 58 strains held for a month, at the end of which time only 46 (79 per cent) gave positive subcultures.

Transfers made from cultures in the glucose medium after incubation for 2 days frequently failed to grow. However, some gonococci may survive longer in the fermented medium, because 25 of 58 yielded subcultures after 4 weeks of incubation and 2 of another group of 14 strains were still viable after 5 weeks.

The 28 strains of meningococci were grown and stored at 37 C. They were maintained without difficulty by subculturing at 3- or 4-week intervals.

*Cultivation of other organisms* In addition to *Neisseria* cultures, 10 freshly isolated strains of *Brucella*, 10 of *Corynebacterium diphtheriae*, and numerous streptococci gave conventional fermentation reactions in the medium containing appropriate carbohydrates. The brucellae and one pneumococcus strain required 2 days of incubation, and the other organisms grew in 1 day. In the absence of the sulfur compounds, the diphtheria bacilli required 8 to 24 hours longer to develop definite reactions. In the medium without fermentable carbohydrate, 4 strains of pneumococci, 2 beta streptococci, 9 *B. abortus*, 1 *Brucella suis*, and 10 cultures of diphtheria bacilli have been maintained at room temperature for more than 2 years by monthly transfers.

*Carbon dioxide requirement* There was no significant difference apparent in cultures grown at the same time upon the casein digest medium with and without reinforcement of the atmosphere with carbon dioxide. However, all of the cultures so tested, which included many gonococci, and all of the meningococcus and brucella strains previously mentioned, had been transferred at least twice after isolation. An experiment was therefore performed to determine whether freshly isolated gonococci would grow in the supplemented casein digest medium without addition of carbon dioxide to the atmosphere. Eighty-five colonies from diagnostic plates were inoculated into the maltose medium, transfers were then made

into glucose medium, and both sets of tubes were incubated in an ordinary incubator. The cultures grew out promptly and characteristic alkaline or acid reactions were visible in 18 hours. This evidence, which indicates that freshly isolated gonococci grown in this medium do not require special provision for exogenous carbon dioxide, is in direct contrast to results obtained with other media. Chocolate agar plate cultures made in duplicate with numerous strains of gonococci (including 20 of the same series) and incubated simultaneously showed little or no growth in 18 hours and rarely had good growth after incubation for 48 hours when no provision was made for reinforcement of the atmosphere with carbon dioxide, control plates, on the other hand, which were incubated in candle jars, regularly showed fair to good growth after 18 hours and always had typical colonies after 48 hours of incubation. Similarly, broth cultures repeatedly failed to develop when tubes were incubated in air, but showed definite turbidity when kept in candle jars for 18 to 48 hours.

#### DISCUSSION

Pancreatic digest of casein in a medium containing both cystine and sodium sulfite has been found capable of supporting the growth of 267 strains of freshly isolated gonococci. This medium, on omission of the sulfur compounds, failed to support the growth of an appreciable proportion (60 to 65 per cent) of the strains tested. The findings indicate the importance of the sulfur compounds as components of the proposed medium, which otherwise is characterized by a low sulfur and cystine content. These observations are in general agreement not only with those of Boor but also with the results reported by Welton, Stokinger, and Carpenter (1944), who found cystine necessary for the growth of stock strains in a defined medium, by Lankford (1944), who incorporated cystine in isolation media to increase colony size, and by Landy and Gerstung (1945), who employed a casein (acid?) hydrolyzate for studying sulfonamide resistance. The apparent discrepancy of the present results with the evidence of cystine inhibition of certain glutathione-requiring stock strains of gonococci, obtained by Gould (1944) and by Gould, Kane, and Mueller (1944), may perhaps be explained by the alteration of metabolism noted during their investigations, or may be, at least in part, due to a presumably higher cystine content, especially when a meat infusion base was employed. Inhibitory action toward gonococci by large amounts of cystine (about 0.09 per cent) was described by McLeod, Wheatley, and Phelon (1927), and they also noted possible stimulation of growth on meat-extract, blood agar medium when lower percentages of the amino acid were used.

The maintenance of gonococci was readily accomplished in the supplemented casein digest medium, especially in the absence of fermentable carbohydrate. Although no attempt was made to establish the exact duration of viability of large numbers of strains in this medium, all of 251 cultures could be carried indefinitely when transferred at intervals of 10 to 14 days, the majority of tested strains remained viable for several weeks without retransfer. The absence of fermentable carbohydrate and consequent acid formation only in part accounts for the prolonged viability of the gonococcus cultures in this medium, since, al



though many 2-day cultures did not yield subcultures, a surprising number survived storage for some weeks. Whatever the factors may be that favor survival or longevity of cultures, it seems clear that freshly isolated gram-negative cocci do not have an inherent tendency to grow and die quickly, and that the viability of a given population may be considerably lengthened under suitable environmental conditions.

Pancreatic digest of casein as a basic nutrient material has several practical advantages. A medium suitable for cultivation of gonococci can be prepared very simply, without the addition of tissue fluids. Its freedom from thermolabile components makes it possible to sterilize the whole medium by autoclaving. It does not require the addition of tryptophane and vitamins, as is the case when the casein has been hydrolyzed with acid, because these substances are retained during enzymatic hydrolysis of the protein. Its freedom from fermentable carbohydrates permits its use as a base in the performance of fermentation tests.

Although tested mainly with gonococci, the supplemented casein digest medium consistently displayed the same desirable characteristics, when used in comparative studies with a limited number of other bacteria.

#### SUMMARY

A simple autoclaved semisolid medium containing pancreatic digest of casein, cystine, and sodium sulfite provided a suitable substrate for the cultivation of freshly isolated gonococci. For the development of cultures in this medium, incubation in an atmosphere reinforced with carbon dioxide was not obligatory.

Gram-negative and gram-positive cocci, brucellae, and diphtheria bacilli could be maintained indefinitely by relatively infrequent transfers.

Accurate fermentation reactions with appropriate carbohydrates were obtained promptly with freshly isolated strains of *Neisseria* and other organisms.

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# FURTHER STUDIES ON THE MORPHOLOGY OF *ESCHERICHIA COLI* EXPOSED TO PENICILLIN

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In a previous paper, Shanahan, Eisenstark, and Tanner (1947) reported electron microscope studies of *Escherichia coli* cells exposed to penicillin. Brief mention was made of light microscope studies in contrast with electron microscope results. Further information gained by light microscope studies as well as other pertinent data is presented in the present paper.

## MATERIALS AND METHODS

Twenty-four strains of gram-negative rods considered to be *Escherichia coli* were used. Strains 251, 252, 253, 254, and 257 were from the department culture collection. Strains "O," "R," and "U" were kindly supplied by Dr. Altme-Werber of the Jewish Hospital, Brooklyn, New York. Eight other strains were obtained from various laboratories, and the remaining eight cultures were isolated by the senior author from fecal samples. Stock cultures were routinely transferred on nutrient agar with frequent colony isolations made to ensure pure cultures.

As suggested by the work of Altme-Werber *et al.* (1945), MacConkey's agar (Difco) was selected as the test medium. Concentrations of penicillin<sup>2</sup> are given in terms of final concentration in the medium. For examination of morphological changes in cells, colonies were selected on MacConkey's agar plates, which were incubated at 37 C. Gram stains and certain other staining procedures were used to determine the morphology after cells had grown in penicillin-containing media. Slide cultures for the examination of living cells were made as follows. About 0.1 ml of MacConkey's agar containing a desired concentration of penicillin was pipetted aseptically onto a sterile glass slide. A small drop of the test organism, usually diluted 1:100 with sterile saline or broth, was placed on the hardened agar. A sterile cover slip was quickly placed over the inoculum and agar. Melted paraffin was run around the edges of the cover slip with a wooden applicator to ensure a firm seal. Culture slides were placed in sterile petri dishes and incubated in a moist chamber at 37 C. Slides were removed for examination at intervals, all examinations being made with oil immersion lens.

Dienes (1942) noted that large round bodies developing in bacterial cultures were fragile, and he did not recommend direct microscopical examination of unstained cultures. In the present work it was found relatively simple to tease off

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<sup>2</sup> Na-penicillin supplied by Schenley Laboratories, Lawrenceburg, Indiana.

cover slips and transfer them to other slides coated with agar, after which they were reincubated. Fusiform structures did not appear to be injured in this process. Single cell isolation was not attempted, but the transfer of cover slips bearing fusiforms was substituted, since single cells could be found in a single field if care was taken in the process.

After cover slips were teased from slide cultures, they were stained with dilute methylene blue or by a modification of Giemsa's staining procedure. In the latter case cells were hydrolyzed for 5 to 10 minutes in  $N$  HCl at 55 C, followed by staining overnight in dilute Giemsa stain. Many specimens were also stained without hydrolysis. Stained preparations were preserved by mounting them in Canada balsam on glass slides.

Photomicrographs were made of stained and unstained cultures. The latter were photographed directly through an oil immersion lens while the organisms were being incubated on a warm slide. A Leitz-Wetzlar "makam" was used for all photographs presented.

#### EXPERIMENTAL RESULTS

The results with several strains of *Escherichia coli* exposed to penicillin were essentially in agreement with those reported by Gardner (1940), Altire-Weber *et al* (1945), Thomas and Levine (1945), Fennel (1946), and others. Sixteen strains were tested several times for growth in various concentrations of penicillin in nutrient broth. Five of the 16 strains would not grow in concentrations above 10 units per ml. Two of the 5 strains repeatedly showed pleomorphism, including elongated swollen rods, filamentous cells, and fusiform structures. The other 3 strains occasionally were pleomorphic and generally became elongated. Eleven strains would grow in broth with 50 units per ml and 2 of the 11 would grow with 100 units per ml, but in no higher concentrations. Pleomorphism was generally evident in the 11 strains, but only in the presence of higher concentrations of penicillin. Strain 257, for example, showed numerous fusiform cells with 100 units per ml but only normal short rods with 5, 10, or 25 units. This merely confirmed earlier evidence that pleomorphism occurred more consistently at sub-inhibitory concentrations of penicillin.

Repeated transfer of several strains of *Escherichia coli* in broth with penicillin showed an increase of resistance to the drug with each passage. As the resistance increased, pleomorphism decreased in lower concentrations and appeared in the higher concentrations of the next passage. Gram stains made from tubes showing little or no turbidity due to high concentrations of penicillin often revealed the presence of pleomorphic forms. Subcultures from tubes containing "debris" but no visible cells usually resulted in the growth of normal organisms on media not containing penicillin but no growth in agar with penicillin of the same concentration as the original cultures. Thomas and Levine (1945) reported a finding of similar sediments with globular masses that yielded normal rods in broth subcultures.

Sixteen strains of *Escherichia coli* tested in broth cultures were exposed to various concentrations of penicillin in MacConkey's agar. Pleomorphism was

evident in all but one strain, and, as in broth, subinhibitory concentrations of penicillin yielded greater numbers of fusiforms and other pleomorphic types. In general, the organisms grew in higher concentrations of penicillin in agar than

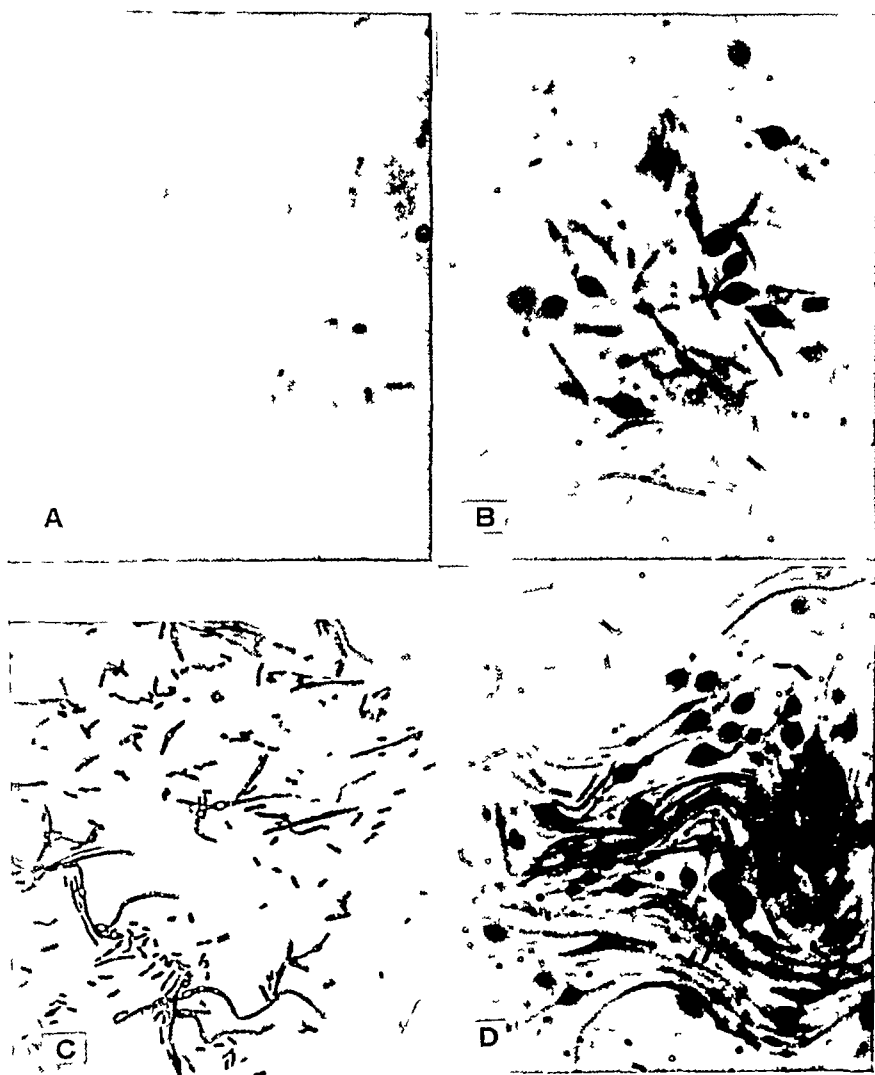


FIG. 1 No. A, gram stain, approximately 3,000  $\times$ , no. B, gram stain, 970  $\times$ , no. C, cold carbol fuchsin stain, 970  $\times$ , no. D, methylene blue stain, cover slip from slide culture, 970  $\times$ .

they did in broth. In the 15 strains that produced morphological variants, the types of cells found were remarkably similar. Among the forms, other than normal rods, were elongated swollen rods, filamentous rods, filamentous rods with fusiform structures usually near the middle of the cell, terminal or lateral

bulb structures, spindle forms, over-shaped cells, and occasionally a large round form. Figure 1, no A, shows a typical fusiform cell appearing in strain 232 with 100 units per ml of penicillin. Figure 1, no B, shows terminal bulb forms of strain A with 50 units per ml, both strains having been grown in MacConkey's agar.

In no case did any culture contain only one form or type of pleomorphic cell. High concentrations of penicillin would often eliminate normal rod cells, as determined microscopically, but a mixture of other cells remained. Subcultures of pleomorphic cells to media without penicillin always gave only normal organisms. Wahlin and Almaden (1939) found a correlation between R and S colonies and the appearance and disappearance of filamentous cells bearing fusiform structures. They felt that the R type colony was a necessary precursor for the appearance of "megalomorphs." No colony differentiation was noted in the present work even when fusiform cells dominated in a colony. It is felt, however, that filament formation does precede the appearance of fusiform structures in a cell.

Organisms from colonies on MacConkey's agar containing penicillin were always gram-negative regardless of their form. Swollen fusiform areas usually stained much darker than the remaining portion of the cells. An unstained area in the center of fusiforms was often visible after gram staining and was especially well brought out with carbol fuchsin. Figure 1, no C, shows the appearance of penicillin-treated cells after they were stained with cold carbol fuchsin. Thomas and Levine (1945) mentioned similar results with this stain. Stains made of old cultures showed a thickening of the fusiform wall and often a demarcation between the fusiform structure and the rod portion of the cell. Granulation of older cultures was discussed previously (Shanahan, Eisenstark, and Tanner, 1947).

Reed and Orr (1923) noted the influence of H ion concentration upon the structure of *Hemophilus influenzae*. They observed that a variety of abnormal cell forms, including fusiform structures, appeared with extremes of acidity and alkalinity of the medium. In the present work it was found that a slight acid condition favored the appearance of fusiforms in the presence of penicillin. Further information on the effects of pH and fusiform cells was obtained following the observance of alkaline reversion on MacConkey's agar by 13 of 17 strains of *Escherichia coli*. Alkaline reversion was characterized in all cases by a change from red to white colonies accompanied by a clearing of the medium. It was soon noted that white colonies no longer contained cells with fusiform structures. Two strains studied in detail were consistent in producing fusiform cells in the presence of penicillin in MacConkey's agar. When red colonies of these strains turned white following alkaline reversion, the abnormal forms were no longer found. It appeared then that there was a correlation between pH and the presence of fusiform cells, although these results are not in agreement with those of Reed and Orr (1923).

Twenty-three strains of *Escherichia coli* were examined in slide cultures prepared by the technique described above. Fusiform cells appeared in all 23 strains when cultured in the presence of various concentrations of penicillin in MacConkey's medium. Since all strains responded in a similar manner, a sum-

may of the growth of fusiform cells may be given. Increasing amounts of penicillin tended to eliminate normal rods, leaving fusiform type cells well isolated in oil immersion fields.

Slide cultures of *Escherichia coli* responded to 50 units per ml of penicillin as follows. After 2 hours' incubation at 37 C cells were either of normal length or double normal length as compared with the control cells. Elongated cells seemed to be more slender than control cells and often showed a tendency to curl or twist upon themselves at this time. Cells not showing elongation proceeded to divide and form microcolonies in the same fashion as control cells. Elongated cells, upon further incubation, failed to divide and became thickened to some extent. This was taken as a criterion that fusiform swelling would occur. Most of the strains required about 4 hours of incubation before fusiform structures became generally evident. A few bulbous structures usually appeared between the second and fourth hours.

First evidence of fusiform swelling, following elongation, was the appearance of two dark areas, half-moon-shaped and approximately in the middle of the cell. The dark areas were on opposite sides of the cell wall and indicated the spot where swelling would occur. Efforts to obtain photographs of cells showing these areas failed. Direct swelling of the cell followed the appearance of these areas that might or might not remain visible in the swollen fusiform. Young fusiform cells were homogeneous internally and became granulated only as the cultures aged.

In the presence of 50 units per ml of penicillin, crowding of the microscope fields usually occurred. With 100 or even 150 units per ml, fields with only fusiform cells could be found readily in most strains. These cells showed little or no tendency to form microcolonies and were thus well suited for observation. Methylene blue staining was satisfactory for demonstrating granules in the fusiform body and often in the rod portion as well. The stain was applied to cover slips teased from the agar cultures. Giemsa stains were not as satisfactory, but differentiation of the bulbous portion and rod portion of a cell was often observed. The fusiform portions varied from light pink to dark purple, whereas rod portions were ordinarily a uniform light blue. Figure 1, no. D, represents a typical field of strain 252 with 100 units per ml of penicillin in MacConkey's agar with cells stained by methylene blue. Strain 251 with 150 units per ml of penicillin is shown in figure 2, no. A. Note the streptobacilluslike appearance of the rod portions brought out by methylene blue stain.

Continued incubation of slide cultures with fusiform cells did not bring any noticeable change except the previously mentioned granulation and autolysis of some cells. Cover slips teased from penicillin agar were transferred to both penicillin and nonpenicillin slide cultures in an attempt to observe any possible further development of fusiforms. In both cases the swollen bulbous forms could be found immediately after transfer and for a short time thereafter. Overgrowth of normal rods ordinarily obscured the fusiforms because of the fact that normal rods were always present on the transferred cover slip. Even with 200 units per ml of penicillin in the agar, normal organisms were found in the liquid at the edges of the agar. This was found to have one advantage in that motility of the

organisms could be readily determined. In all but a few cases cell motility was evident with control cultures and in cells bearing fusiform bodies. The motility

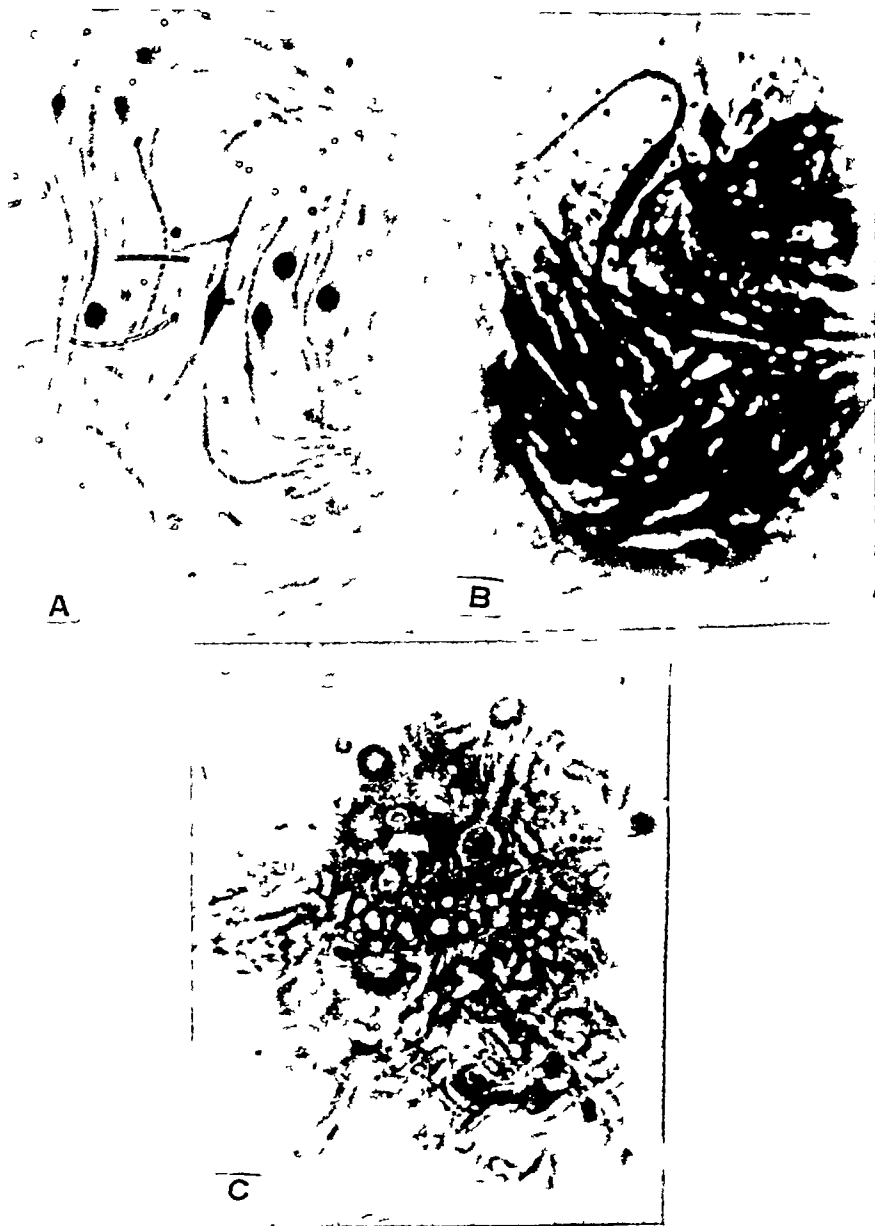


FIG. 2. No. A, methylene blue stain, cover slip from slide culture, 970 $\times$ ; no. B, unstained, living cells, 970 $\times$ ; no. C, unstained, living cells, 970 $\times$ .

of the latter cells was rather peculiar in that progression was by a sluggish undulating motion of the entire cell. The edge of an agar slide culture of strain 293 is shown in figure 2, no. B. Note the large spindle form near the upper middle.



Preliminary plating of several strains of *Escherichia coli* in lactose agar with various concentrations of penicillin indicated that fusiform structures did not appear with the same frequency as in MacConkey's medium with penicillin. Lactose agar slide cultures of strains 252 and "O" revealed a type of cell not previously encountered. After 3 to 4 hours' incubation at 37 C many large round cells could be found, and, although they resembled the fusiform structures in size and shape, rod portions seemed to be absent. Such cells were photographed with difficulty because of the clarity of the medium (figure 2, no. C). Round cells were often indistinct in living cultures, and stained preparations were not satisfactory. These forms appear to be similar to those reported by Kuhn (1924), Hussong (1933), Dienes (1939, 1942, 1946), and Stubblefield (1947) among others.

#### DISCUSSION AND SUMMARY

Wahlin and Almaden (1939) presented a list of those authors who, they considered, had been dealing with true "megalomorphs" or fusiform cells. To this list should be added the following: Ohlmacher (1902), Peju and Rajat (1906), Wilson (1906), Scales (1921), Reed and Orr (1923), Stuart (1924), Tetrault (1930), Cunningham (1931), Kritschewski and Ponomarewa (1934), and Price *et al.* (1947). Fusiform structures in cultures exposed to penicillin were reviewed by us earlier (1947). The list given above does not include papers dealing with fusiform structures encountered in pleuropneumonia-like organisms. The similarity of bacterial fusiforms and the organisms described by Parker and Hudson (1926), Klieneberger (1934, 1935), Dienes (1939, 1942, 1946), and others seems quite evident.

Dienes (1946) held that fusiform cells produced under toxic influences usually were not viable. Fusiforms occurring in cultures under normal conditions apparently develop further as shown by Dienes in numerous papers. In the present studies fusiform cells were not observed to undergo further development according to methods employed. The medium as well as penicillin may have some effect on fusiform formation and viability.

Twenty-four strains of *Escherichia coli* were exposed to various concentrations of penicillin in nutrient broth, in MacConkey's agar plates, and on slide cultures. All strains responded under one condition or another by showing formation of fusiform cells. On lactose agar slide cultures, a somewhat different type of cell was noted in two strains exposed to penicillin. The mechanism of fusiform growth was observed under oil immersion lens, and a description of the techniques employed is given. Photographs are presented showing abnormal forms of *Escherichia coli* encountered when penicillin was incorporated in the medium.

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# A MICROBIOLOGICAL METHOD FOR THE ASSAY OF SUBTILIN

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A turbidimetric method for the assay of subtilin has recently been reported by Lewis *et al.* (1947). In our laboratory a microbiological method, based on the principles described by Vincent and Vincent (1944) utilizing filter paper disks on agar plates, has served as a satisfactory assay for subtilin. The details of this method and the effect of various physical and chemical factors on the results obtained from it are described below.

## PROCEDURE

*Medium and preparation of plates* The medium used in the assay is composed of Difco peptone 0.5 per cent, beef extract 0.2 per cent, yeast extract 0.3 per cent, NaCl 2 per cent, and agar 1.5 per cent. These ingredients are dissolved in distilled water, and the pH is adjusted to 6.4 with HCl. After sterilization at 15 pounds' pressure for 15 minutes the medium is allowed to cool to 50 C, and 20-ml amounts are distributed to petri plates. A 20-ml syringe equipped with an automatic double valve (BD no. 470V) has been found to be convenient for this operation. After the agar solidifies, each plate is layered with 5 ml of the same agar seeded with the test organism. The plates are stored in the refrigerator as soon as they solidify.

Inconsistent results are obtained when plates are used that have been seeded for more than 4 days. If assays are to be run over a longer period, a sufficient number of plates containing the initial 20 ml of agar is prepared and the plates are then seeded as needed. These plates are stored in the refrigerator. More uniform seeding of the plates occurs if they are allowed to come to room temperature before seeding. Plates seeded at two different times should not be mixed in running assays.

*Test organism* Stock spore suspensions of *Bacillus cereus* 247 are prepared by growing the organisms on the surface of Difco nutrient agar for 4 days at 34 C. The organisms are washed twice in distilled water, heated at 50 C for 30 minutes to kill the vegetative cells, and the suspensions stored in the refrigerator. The agar overlay should contain 300,000 spores per ml.

*Preparation of samples* A solution of the reference standard which is twice the maximal concentration used to obtain the standard curve<sup>2</sup> is prepared by

<sup>1</sup> With the technical assistance of A. S. Herring.

<sup>2</sup> With the sample of subtilin used in our work this maximal concentration was 1,000 µg per ml. Since there seems to be no generally accepted unit, and since each sample will vary in potency, it is impossible to indicate the maximal concentration. It is necessary, therefore, to determine this concentration with each sample of subtilin by preliminary experiments.

The subtilin used in this work was a single lot (no. 118-T dated Aug. 19, 1946) furnished

dissolving the dry preparation in 1 N acetic acid. This solution is stored in an amber glass bottle in the refrigerator, since subtilin has been shown to decrease in potency in the presence of light (Jansen and Hirschmann, 1944). It is thus necessary to limit exposure of both the unknown and the standard. On the day of the assay a portion of the stock solution is brought to pH 5 with 1 N NaOH, and sufficient 0.2 M Soerensen's phosphate buffer (pH 5.8) is added to give a final subtilin concentration equal to that of the most concentrated sample to be used on the standard curve. This results in a buffer concentration of approximately 0.1 M. Further dilutions of the standard are made with 0.1 M buffer. It is recommended that five concentrations be run, spaced over the 10-fold concentration range of the assay. All unknowns are prepared so that the final buffer concentration is 0.1 M and the pH is 5.8. Alterations in the procedure for assaying subtilin in blood or serum are discussed in a later section.

Sterility is not required in the preparation of the unknowns or in the dilutions of the standard.

*Setting up the assay.* It is recommended that each dilution of the standard and the unknown be run in triplicate. The assay is set up by placing 3 filter paper disks,<sup>3</sup> flat side down, on 3 agar plates and pipetting a 0.10 ml aliquot of the sample onto each one as rapidly as possible. Only 3 disks are used on each plate because of the size of the zones obtained with high concentrations. A standard curve is run each time that unknowns are assayed.

*Incubation of the plates.* Plates are incubated at 30°C for 18 to 24 hours, and the diameters of the zones of inhibition are read to the nearest 0.5 mm. Shorter periods of incubation at 37°C are satisfactory, but the zones of inhibition are smaller than on plates incubated at 30°C.

*Estimation of potency and its error.* To illustrate the manipulation of results obtained in determining the estimate of potency of an unknown and its error, an example is given below.

Figure 1 shows the relationship between zone diameter and log of subtilin concentration under the experimental conditions proposed above for routine assay. This is a typical standard curve, except that 11 dilutions each in 6 replicates were run. The points on the curve represent the averages of the replicates. By plotting zone diameters versus log concentration the relationship becomes linear over the range of concentrations indicated. The line in figure 1 was computed by the method of least squares ( $y$  on  $x$ ). For rigorous statistical treatment of the data this method is necessary, but for practical purposes it was found that the graphical method (using transparent ruler) suffices, provided the one who draws it has some experience with the method. Transforming the data to give linear regression<sup>4</sup> has the following advantages: (1) the best estimate of the correlation

by Dr. H. D. Lightbody, Western Regional Research Laboratories of the U. S. D. A., Albany, California. On the basis of a unit which has been proposed by Sallé and Jann (1946) this sample contained about 1 unit per 100  $\mu$ g dry weight.

<sup>3</sup> Schleicher and Schuell no. 740 E  $\frac{1}{2}$ -inch filter paper disks.

<sup>4</sup> One cycle semilogarithmic paper can be used, thus eliminating the necessity of transforming concentrations into logarithms.

line is available, and (2) the error of the method can be calculated. The broken lines in figure 1 parallel to the solid line represent confidence limits for single zones spaced 2 standard errors of the estimate from the solid line. This means that 19 times out of 20 the zone size obtained by any specific concentration  $x$  should fall within these limits. The confidence limits are actually parabolic, but in this case the error introduced by assuming them to be linear is very small.

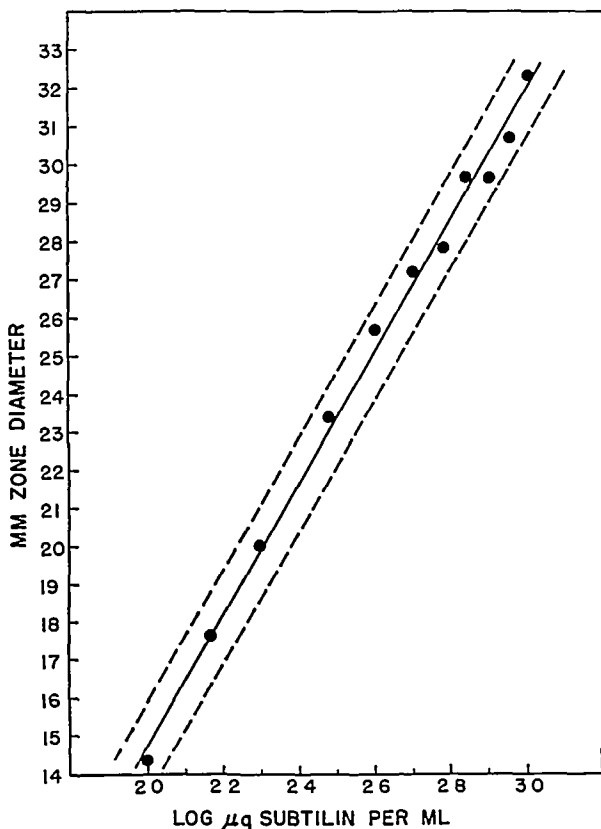


FIG 1 STANDARD CURVE FOR THE DISK ASSAY OF SUBTILIN

The variance around the line over the range of concentrations indicated was found to be homogeneous by Bartlett's Chi-square test (Snedecor, 1946).

Taking figure 1 as the standard curve, an unknown (1 disk) falling within the range will have an error of  $\pm 17$  per cent<sup>5</sup>. It is proposed as standard procedure in this assay to run all dilutions of the antibiotic in triplicate. The diameters of the zones are averaged, and the estimate of concentration is taken from the aver-

<sup>5</sup> Error limits are given as  $\pm 2$  standard errors, which means that 19 times out of 20 the true values will be within these limits. The upper and lower confidence limits are not exactly equal when converted to antilogs, but for practical purposes they are made equal by taking their average.

age. If one dilution falls within the range, the error is  $\pm 10$  per cent. When more than one dilution falls within the range, an estimate of concentration is obtained from each dilution and the estimates are averaged. The errors for 2, 3, 4, and 5 dilutions falling within the range are  $\pm 7$ ,  $\pm 7$ ,  $\pm 9$ , and  $\pm 4$  per cent, respectively.

Although the error of the method should not vary too much if the technique is well controlled, it is advisable that each laboratory determine its own error for the method and perhaps check it occasionally.

#### EFFECT OF VARIABLES

*pH of the medium.* In the early portion of the work NaCl was not included in the assay medium. During this period some experiments were done to deter-

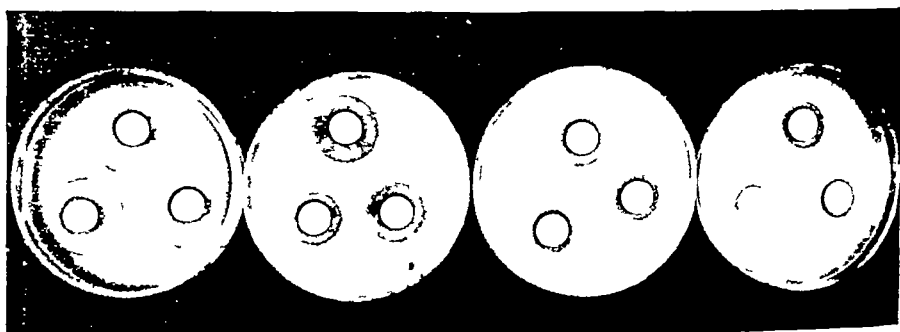


FIG. 2. EFFECT OF pH OF THE MEDIUM ON THE ZONE OF INHIBITION.

pH of medium in plates, left to right, is 5.9, 6.2, 6.4, and 7.2, respectively. Top disk on each plate moistened with 0.10 ml subtilin containing 1,000  $\mu\text{g}$  per ml, right disk 500  $\mu\text{g}$  per ml, left disk 333  $\mu\text{g}$  per ml. Zones of inhibition obtained at pH 5.9 are outlined in ink. The poor growth at this pH is not visible on the photographic plate. No NaCl in the medium.

mine the effect of the pH of the medium on the activity of subtilin. The results of one such experiment are shown in figure 2. Maximal activity occurred at pH 5.9, and there was a gradual decrease in activity as the pH was increased to 7.2. The exceptionally large zones of inhibition observed at pH 5.9 were probably due largely to inhibition of growth of the test organism by the low pH. Above pH 6.2 there was no obvious change in density of growth. Although larger zones were obtained at pH 6.2 than 6.4, the latter pH was chosen for routine assay to permit a factor of safety in the adjustment of the pH of the medium.

*NaCl concentration of the medium.* Larger zones of inhibition were observed when NaCl was included in the test medium. In this series of experiments the medium was adjusted to pH 6.4 and incubated at 30 C overnight. Control were run with no NaCl, and similar assays were done on media containing 0.5, 2, and 5 per cent NaCl. The test organism did not grow on the medium containing 5 per cent NaCl. The size of the zones varied directly with the concentration of NaCl (figure 3). On the basis of these results, 2 per cent NaCl was included in the media employed throughout the remainder of the work.

*Assay of subtilin in certain body fluids.* Larger zones of inhibition were

served when subtilin was assayed in the presence of blood or serum, as compared with those obtained in the presence of phosphate buffer. It was found that within certain limits, in the presence of a constant amount of subtilin, the zone diameter was dependent on the amount of blood and serum present.

Consistent results were obtained in the presence of more than 10 per cent blood or more than 12 per cent serum. When 100  $\mu$ g subtilin per ml were assayed in the presence of varying concentrations of serum (0.05 to 50 per cent), there was a difference of 10 mm in diameter of the zones over the range of 0.05 per cent to 12 per cent serum, and negligible differences in higher concentrations. These results could not be duplicated in the presence of human globulin or albumin alone or in combination.

In view of the foregoing results it is necessary, when assaying for the antibiotic

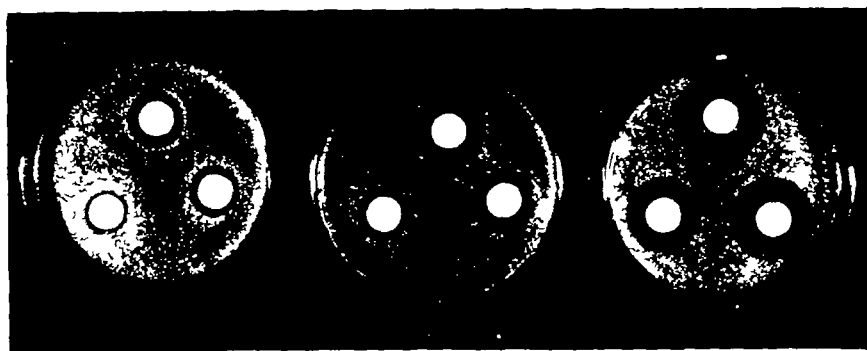


FIG. 3. EFFECT OF NaCl CONCENTRATION OF THE MEDIUM ON THE ZONE OF INHIBITION

NaCl concentration in the plates, left to right, is 0, 0.85, and 2 per cent, respectively. Top disk on each plate moistened with 0.10 ml of subtilin containing 1,000  $\mu$ g per ml, right disk 500  $\mu$ g per ml, left disk 333  $\mu$ g per ml. pH of medium 6.4.

in blood or serum by this method, to dilute the unknown and the sample used for the standard curve with the concentrations of blood or serum mentioned above. The observation that the zone diameter was consistent when different blood or serum samples were used permits dilution of the unknown and standard with normal blood or serum.

The assay of subtilin is not affected by the presence of normal urine in concentrations up to 50 per cent. Assays of such samples are computed from the standard curve obtained from samples diluted with 0.1 M phosphate buffer at pH 6.4.

#### SUMMARY

A filter paper disk method for the microbiological assay of subtilin, using *Bacillus cereus* 247, has been described. This method also is applicable to the assay of subtilin in certain body fluids. With the proposed procedure the error of the assay is approximately  $\pm 5$  to 10 per cent, depending on the number of dilutions of the unknown falling within the range of the standard curve.

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# PIGMENT PRODUCTION BY *NEUROSPORA CRASSA* IN THE PRESENCE OF PARA-AMINOBENZOIC ACID<sup>1</sup>

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Recent investigations of altered sulfonamide responses in *Neurospora crassa* (Emerson and Cushing, 1946) have disclosed that high concentrations of *p*-aminobenzoic acid (PABA) depressed and even inhibited the growth rate of various strains studied. The present paper reports observations upon a pigment produced by *Neurospora crassa* when growing in "minimal" medium (Beadle and Tatum, 1945) supplemented with  $10^{-2}$  moles of PABA. Specific interest, beyond a general relation to studies of PABA metabolism, may be attached to these observations because of work showing that a strain of *Mycobacterium tuberculosis* (Mayer, 1944) also produces a pigment when growing in the presence of this factor. In addition, Mayer notes that *Escherichia coli* and *Aspergillus niger* produce pigments under similar conditions, and Spink and Vivino (1943) have found that various sulfonamides induce the formation of a pigment by resistant strains of staphylococci, which they believe is derived from the excess PABA produced by these strains. These various pigments, though seemingly related because of their specific association with PABA, differ among themselves and from the one described here.

The fact that the toxicity of PABA in high concentrations extends to such diverse organisms as dogs (Scott and Robbins, 1942), and to microorganisms (Cavill and Vincent, 1945) other than *Neurospora* lends additional interest to a consideration of the colored compounds produced in its presence. In this regard, Cavill and Vincent also note that the molds *Aspergillus*, *Penicillium*, and *Byssoschlamys* form an orange-yellow pigmentation in the mycelial felt and in the surrounding medium when growing in high PABA concentrations, but do not report further on this coloration.

The present experiments, unless otherwise noted, were conducted with several strains of wild type *Neurospora crassa* grown at 30 C on liquid medium (20 ml in 125-ml Erlenmeyer flasks). When this medium consisted of "minimal" supplemented with  $10^{-2}$  moles PABA (pH adjusted to 5.5 at the start of the experiment and changing to 4.2 to 3 as growth progressed), the growth rate of *Neurospora* was depressed, as noted earlier. As growth proceeded, a green pigment gradually appeared in the vicinity of the mycelium and accumulated until, by 78 hours, the culture fluid was greenish yellow. By the end of 6 days full growth of the mycelium had been reached and abundant conidia produced. At this

<sup>1</sup> The assistance of a research grant from the U. S. Public Health Service is gratefully acknowledged.

<sup>2</sup> The authors wish to thank Miss Theodora Penn for technical assistance during this work.

time the medium was conspicuously orange red in color. Separation of the mycelial mat by filtration showed this color to be due to a material that had accumulated in the hyphal strands, the medium itself remaining greenish yellow. Flasks containing the PABA medium, but not inoculated, remained colorless, showing that the pigments are a product of *Neurospora* metabolism. Concentrations of  $10^{-1}$  moles of PABA completely inhibited growth, but did develop a greenish tinge spontaneously. This product, however, was found to have quite different properties from those described for the material produced by the mold. Growth in concentrations of PABA =  $10^{-3}$  moles or lower produced no pigmentation.

In addition to wild type strains from several sources, grown at 35 C as well as at 30 C and on solid as well as liquid media, the following mutant strains also produced the colors described: *p*-aminobenzoicless 15835a (from the California Institute of Technology) and the sulfanilamide-resistant (C-40) and sulfanilamide-requiring (E-15172A) strains described elsewhere (Emerson and Cushing, 1946, Emerson, 1947). In addition, an albino strain (12-2) obtained in this laboratory also reacted as did the wild type with respect to the production of pigment, although it retained its albino characteristics.

An attempt was made to obtain genetic alterations in the response of *Neurospora* through adaptation. To this end, four parallel series of serial transfers of cultures growing on  $10^{-2}$  moles of PABA were started, but at the end of 4 months representing 8 such transfers no detectable deviations from the wild type behavior in pigment production or growth rate were found.

That pigment production is specifically associated with PABA is shown by the fact that like concentrations of several amino acids and vitamins (M. Schwartz, unpublished data) added individually to minimal medium had no effect, even on the growth rate, upon wild type *Neurospora*. In addition, neither benzoic nor anthranilic acids stimulated pigment production, for these either inhibited growth at  $10^{-2}$  moles or gave colorless growth at lower concentrations.

The effects of PABA were not altered in media containing, in addition to  $10^{-2}$  moles of this factor, mixtures of the vitamins or the amino acids normally used in this laboratory for testing for biochemical mutations in *Neurospora* and present in the concentrations used for these tests (McElroy, Cushing, and Miller, 1947).

The pigment was also produced when glucose was substituted for sucrose. This is different from the reactions of *Mycobacterium* when glucose is substituted for glycerol (Mayer, 1944).

The relationship between the yellow-green pigment occurring in the medium and the orange-red pigment in the hyphae has not been established, and it has been convenient to consider the two occurrences separately. The results reported below are concerned only with the pigment found in the filtrate. The material has not yet been obtained in crystalline form, the best preparations being amorphous orange-red powders.

Extraction of the colored material is best done with the concentrated filtrate.

at pH 4 and peroxide-free ether, lesser yields are obtained at pH 1 and pH 11. The substance is readily soluble in the lower alcohols, and may be extracted from the concentrated filtrate with *n*-butyl alcohol. Hydrocarbons, e g, hexane, benzene, toluene, as well as chloroform and carbon tetrachloride, do not extract the pigment from aqueous solution. However, high specific solubility is shown by the dried material in that, whereas it is insoluble in carbon tetrachloride and the hydrocarbon solvents, it is soluble in chloroform.

The amorphous powder mentioned above was obtained from toluene solution as follows. Excess toluene was added to the ether extract of the pigment, and the ether was removed by distillation, during which process some insoluble material formed. Hot filtration followed by chilling of the clear green toluene filtrate produced a flocculent reddish precipitate, which appeared to darken somewhat on drying in air. The intensification of color, however, appeared not to be due to any change in the nature of the pigment since dilute aqueous and ethereal solutions of the dried material seem identical with solutions of the original pigment in the same solvents.

The substance is soluble in both acids and bases and can be completely removed from ether-hexane solution by shaking either with cold 20 per cent hydrochloric acid or dilute sodium bicarbonate solution. Redissolution in ether from aqueous solutions, without preliminary concentration, is practical only at pH 4.

Characteristic color changes are noted with change in pH. In dilute bases the color is green yellow, acidification causes a deepening of the green color. Evaporation of the alkaline solution results in golden-yellow residues. Evaporation of acidic solutions formed dark red rustlike residues. Dissolved in concentrated hydrochloric or nitric acids, almost colorless solutions are obtained. Evaporation leaves residues similar to those obtained from dilute acids.

A color test which has been found to be very characteristic of the pigment is conducted as follows. One drop of concentrated hydrochloric or nitric acid is placed on a dry film of the substance. As solution takes place, a purple ring is noticed at the periphery of the expanding drop, which itself remains colorless. Evaporation to dryness leaves the red granular substance mentioned above, which dissolves in a few drops of concentrated ammonia water to form a colorless solution.

The pigment can be adsorbed on norit A from ether-hexane solution, and eluted with alcohol, hot water, acetone, and ammonia-alcohol. The water eluate is opaque to ultraviolet light.

The foregoing data, though preliminary in nature, are presented to give some description of the pigment and the conditions for its formation in order to show that it differs in its properties and mode of appearance from the material described by Mayer. By similar comparisons it has been possible to show that the pigment is neither riboflavin (preparation from Merck) nor folic acid (preparation obtained from General Biochemicals, Inc.). Sufficient properties of the pigment described by Spink and Vivino are not available to permit a satisfactory comparison with those reported here.

## SUMMARY

Wild type strains of *Neurospora crassa* have been found to produce a water soluble greenish-yellow pigment in the presence of  $10^{-2}$  moles of PABA as growth proceeds and to concentrate an orange pigment in their hyphae. Conditions for the production of this pigment are presented, and a procedure is described for the isolation of the crude material. In addition, a color test is described, and sufficient properties are listed to show that it differs from the pigment found by Mayer (1944).

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# THE EFFECT OF PENICILLIN ON THE GROWTH OF MYCOBACTERIUM TUBERCULOSIS IN DUBOS' MEDIUM<sup>1</sup>

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The *in vitro* study of antibiotics for activity against *Mycobacterium tuberculosis* has been hampered by the lack of a convenient method for rapid cultivation of the organism. In the case of penicillin, instability of the antibiotic in solution presents an additional handicap. Abraham *et al* (1941) reported that 1,000-fold the amount of penicillin adequate to produce complete inhibition of a strain of *Staphylococcus aureus* did not inhibit the growth of *M. tuberculosis* in glycerol broth, even though penicillin solution was added every 2 days to maintain the original concentration of antibiotic. The penicillin preparations then available could not be accurately standardized, but the maximal concentration used by these investigators appears to have been in the range of 40 units per ml.

Smith and Emmart (1944) obtained good growth of *M. tuberculosis* human type, strain A27, in glycerol broth containing 10 to 30 units of penicillin per ml and in Proskauer and Beck's medium containing 0.5 to 10 units per ml. The effect of higher concentrations was not studied. Friedmann (1945) obtained rapid growth of human, bovine, and avian strains in tissue cultures containing 12 to 20 units of penicillin per ml, which had been added to inhibit the growth of contaminants.

Recently, Iland (1946), using a rapid slide culture technique, found that small inocula of freshly isolated strains were inhibited by concentrations of penicillin varying from 20 to 60 units per ml. It was his opinion that this sensitivity to penicillin may be lost upon prolonged cultivation of the organisms on artificial media, but this thesis was not tested with virulent laboratory strains. Ungar and Muggleton (1946) studied the effect of concentrations up to 5 units per ml and reported enhanced growth of the organisms in the presence of these concentrations of penicillin. A single strain was used in this study.

Youmans (1944*a,b*) has described an improved medium for testing bacteriostatic agents with *M. tuberculosis*. Subsurface growth is regularly obtained with inocula of 0.1 mg of organisms per 10 ml of medium, but subsurface growth is not regularly obtained with smaller inocula. Dubos (1945) has introduced a rapid tube culture method in which growth is characterized by diffuse turbidity and may be initiated with small inocula. This medium has been used success-

<sup>1</sup> A preliminary report of this study was presented before the Theobald Smith Society on June 21, 1947. Kirby and Dubos, *Proc Soc Exptl Biol Med*, 66, 120, 1947, have since shown that high concentrations of penicillin produce partial lysis of *M. tuberculosis* and that small inocula of organisms in tween albumin medium are susceptible to low concentrations of penicillin. No inhibition was obtained with oleic acid-albumin medium.

fully by Steenken (1946) and by Smith (1947) to study the effect of streptomycin on *M. tuberculosis*

The present study was undertaken to supply additional data regarding the positive effect of penicillin on *M. tuberculosis*. The strains used included 4 recently isolated from active human cases in addition to standard laboratory strains of human, bovine, and avian type. Dubos' medium was selected because it supported rapid and diffuse growth. Since cultures in this medium may be initiated with small numbers of cells, the study of the effect of inoculum size on sensitivity to penicillin was facilitated.

#### MATERIALS AND METHODS

*Strains*<sup>2</sup> MT1, MT2, MT4, MT5, H37Rv (human type), Ravenel (bovine type), and Kirchberg (avian type) were studied. The MT strains were isolated from human sputum approximately 6 months prior to these studies. All strains have been maintained both in Dubos' medium and on Petragnani's medium.

*Penicillin* Crystalline penicillin G sodium (Merck) was used for all tests.

*Preparation of cultures* The culture medium was prepared according to the formula of Dubos and Davis (1946). "N-Z case" (batch 372, Sheffield Farms Company) was selected as the source of nitrogen, and albumin (bovine V, Armour) was used for detoxification of fatty acids. The albumin was dissolved in 0.85 per cent sodium chloride, neutralized, inactivated at 55 C for 30 minutes to destroy residual lipase, and sterilized by passage through a Seitz filter. Penicillin solutions were prepared in Dubos' medium at tenfold the final concentration desired, and 1-ml amounts of penicillin in solution were added to 9 ml amounts of Dubos' medium in 20-by-150-mm culture tubes.

Test and control tubes were inoculated with 0.1-ml amounts of cell suspension. The turbidity of inocula was adjusted, using a spectrophotometer (Coleman model 6A), to a density that permitted 89 to 91 per cent transmission of light at 620  $\mu$ , through suspensions in 19-by-150-mm cuvettes. Suspensions of this turbidity contained approximately  $10^7$  to  $10^8$  viable organisms per ml as determined by a tube dilution culture method. When further dilutions were desired, Dubos' medium was used as the diluent.

Tenfold dilutions ranging from  $10^0$  to  $10^3$  were prepared from cell suspension, standardized as described above. The undiluted and diluted suspensions were then inoculated into series of culture tubes containing 1 to 200 units of penicillin per ml, and growth was determined after 14 days of incubation at 37 C.

#### EXPERIMENTAL RESULTS

The results of 6 consecutive trials in which 4 to 7 strains were used are presented in table 1. With undiluted inocula the strains recently isolated from human cases of active tuberculosis were inhibited by concentrations of penicillin ranging from 80 to 200 units per ml. Among the standard laboratory strains, H37R

<sup>2</sup> Strains MT1, MT2, MT4, and MT5 were obtained from Dr. D. W. Richards, Jr., Bellevue Hospital, New York, N. Y. Strains H37Rv, Ravenel, and Kirchberg were obtained from Dr. W. Steenken, Jr., of the Trudeau Foundation, Saranac Lake, N. Y.

(human type) was inhibited by 100 to 150 units, whereas Ravenel (bovine type) and Kirchberg (avian type) were inhibited by 40 units. In 1 of 4 trials the Kirchberg strain was inhibited by 20 units.

TABLE 1

*Concentrations of penicillin, units per ml, required for inhibition of growth of M. tuberculosis*

STRAIN	DILUTION OF INOCULUM	TRIAL NUMBER					
		1	2	3	4	5	6
MT1	Undiluted				80	100	80
	$10^{-1}$				60	60	80
	$10^{-2}$				40	40	40
	$10^{-3}$				20	20	10
MT2	Undiluted	200	100	200*	100		100
	$10^{-1}$	150	100	100	60		100
	$10^{-2}$	60	40	100	20		40
	$10^{-3}$	40	10		20		10
MT4	Undiluted				200	200	150
	$10^{-1}$				150	100*	80
	$10^{-2}$				60	60	20
	$10^{-3}$				20	60	20
MT5	Undiluted				150	200	100
	$10^{-1}$				40	80	20
	$10^{-2}$				20	20	10
	$10^{-3}$				—	10	—
Ravenel	Undiluted	40	40	40			40
	$10^{-1}$	20	10	10			10
	$10^{-2}$	10	x	x			—
	$10^{-3}$	1	1	x			—
H37Rv	Undiluted	150	100	150	150	150	150
	$10^{-1}$	80	40	60	80	80	60
	$10^{-2}$	40	20	40	40	40	40
	$10^{-3}$	10	x	x	10	10	10
Kirchberg	Undiluted	40	40	40			20
	$10^{-1}$	10	10	10			10
	$10^{-2}$	10	1	x			1
	$10^{-3}$	1	1	1			1

— = no growth in the control without penicillin

x = contaminated

\* Late growth at this concentration, higher concentrations of penicillin were not included in this series

With more dilute inocula lower concentrations of penicillin were required to inhibit growth. At the  $1:10$  dilution the recently isolated human strains were inhibited by 20 to 150 units, H37Rv by 40 to 80 units, and Ravenel and Kirch-

berg by 10 to 20 units. At the 1:100 dilution the inhibiting concentrations, with one exception, varied from 1 to 60 units, and at the 1:1,000 dilution the inhibiting concentrations, with 2 exceptions, dropped to 20 units or less.

#### DISCUSSION

The use of Dubos' medium to support growth of *M. tuberculosis* offers a convenient method for the study of the *in vitro* action of antibiotics, in this case penicillin, on the organism. Growth is relatively rapid and produces a diffuse turbidity which permits standardization of inocula by use of turbidimetric methods. This eliminates the trituration of cell masses for preparing homogeneous suspensions and the need to express the concentration of cells in gravimetric units. Furthermore, the medium consistently supports the growth of small inocula of cells and can therefore be used for the estimation of the number of viable organisms in cell suspensions with a tube dilution culture method.

Depending upon the concentration of organisms in the inoculum, strains of human, bovine, and avian types of *M. tuberculosis* may with few exceptions be inhibited by concentrations of penicillin varying from 1 to 200 units per ml. These results are comparable with those reported by Iland (1946) for freshly isolated strains of the human type. He postulated, however, that sensitivity to penicillin may be lost upon prolonged cultivation of the organisms and cited the high resistance of strain 607, an avirulent strain of human type, as an example. Other standard laboratory strains were not investigated.

Our results indicate that Iland's conclusions, drawn from observations with strain 607, do not apply to several other laboratory strains. Using an inoculum of 0.1 ml containing approximately  $10^6$  organisms, strain H37Rv was inhibited by 100 to 150 units per ml. This value is in the same range of concentrations required to inhibit the group of 4 recently isolated strains used in this study. Two other laboratory strains, Ravenel and Kirchberg, were inhibited by 40 units per ml, which approaches the lower limit of sensitivity observed by Iland. The high resistance of strain 607 appears to be a characteristic of the strain rather than the species. Furthermore, strain 607 is unusual in other respects since, unlike virulent strains, it may be grown on nutrient agar medium.

#### SUMMARY

Depending upon the size of inoculum, human, bovine, and avian types of *Mycobacterium tuberculosis* were inhibited by concentrations of penicillin varying from 1 to more than 200 units per ml. When inocula were adjusted to permit 90 per cent transmission of light at 620 m $\mu$  through a depth of 19 mm, representing an inoculum of approximately  $10^6$  organisms per 10 ml of test medium, growth of the tubercle bacillus was inhibited by drug concentrations of 10 to 200 units per ml.

Lower concentrations of penicillin were effective when smaller inocula were used. With a 1:10 dilution of standard cell suspension as inoculum, half the original inhibiting concentration frequently produced complete inhibition of visible growth. This trend was maintained with higher dilutions of inocula.



Among the human strains, no marked difference in sensitivity was observed between a standard laboratory strain (H37Rv) and strains recently isolated from sputum

The strains of bovine and avian type were more sensitive to penicillin than human ones

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# A NEW SALMONELLA TYPE SALMONELLA WAYCROSS

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The culture was isolated in the Waycross Branch Laboratory of the Department of Health, State of Georgia, and sent to us by Miss Jamie Morris, Atlanta, Georgia. It was recovered from the urine of a woman with a history of pyuria due to chronic cystitis and renal calculi. The patient had fever from time to time.

**Biochemical features** Gram-negative rods that promptly ferment, with gas formation, glucose, mannitol, maltose, trehalose, sorbitol, arabinose, rhamnose, and xylose. They grow on citrate agar and utilize *d*-tartrate on the second day. Lactose, sucrose, salicin, dulcitol, and inositol are not fermented within 3 weeks, indole is not formed, and gelatin is not liquefied (60 days).  $H_2S$  is produced.

**Mouse pathogenicity** Six mice fed with the organism were alive after 28 days. *Salmonella waycross* was found in pellets taken on the ninth and twenty-first days. Of 3 mice injected sc with 0.1 ml of a 24-hour broth culture, 2 died within 24 hours, 1 survived 28 days. Three mice were injected ip with 0.1 ml of a 24-hour broth culture, 2 died within 24 hours, 1 was alive after 28 days. Thus the virulence for mice is relatively low.

**Serology** Dr. Edwards, to whom we had sent the culture, informed us that its O antigen seemed to be identical with that of N J 4, a coliform bacterium isolated from arthritic wing joints of canaries (Edwards, Cherry, and Bruner, 1943). This specific O antigen exhibited a slight relationship in the form of cross reactions to types containing the XI antigen (homologous agglutination, 1:4,000, cross reaction, 1:500). The flagellar antigen of N J 4 was related to the g of the enteritidis group. Dr. Edwards kindly supplied a transplant of N J 4 and thus enabled us to produce sera and antigens for comparative agglutinations and reciprocal absorption tests.

From table 1, the following conclusions may be drawn. *S. waycross* and N J 4 are closely related, the main antigen shows only minor quantitative differences with unabsorbed sera. In absorption tests the two antigens do not fully exhaust the reciprocal antisera. The possible contents of XI as a partial antigen are larger in N J 4 than in *S. waycross*. This is demonstrated by quantitative agglutinations as well as by the far more effective absorption of N J 4 serum by *S. rubislaw* (XI).

Since the N J 4-waycross main antigen so far has not been found in the *Salmonella* group, a new symbol is to be allotted to it. XLI was chosen in agreement with Edwards and Kauffmann. The addition to the formula of the partial antigen XI seems not to be warranted because of its very limited cross reaction with our strain.

The H antigen of *S. waycross* belongs to the  $z_4$  group. "Antigenic relationships within this group are quite complicated," to quote Edwards, West, and Bruner (1947). These antigens occur in paracolon bacilli as well as in *Salmonella* types. They have the  $z_4$  in common and differ in partial antigens, e.g.,  $z_{25}$ ,  $z_{26}$ ,  $z_{32}$ . Some even more individual factors may occur with no particular

TABLE 1  
Somatic relationships of *S. waycross*

ANTIGENS	SERA											
	N J 4				<i>S. waycross</i>				<i>S. rubislaw</i> (VI)			
	Unabsorbed	Absorbed with			Unabsorbed	Absorbed with			Unabsorbed	Absorbed with		
		<i>S. waycross</i>	<i>S. rubislaw</i>	<i>S. waycross</i> + <i>S. rubislaw</i>		N J 4	<i>S. rubislaw</i>	N J 4 + <i>S. rubislaw</i>		<i>S. waycross</i>	N J 4	N J 4 + <i>S. waycross</i>
N J 4	640	20	160	0	800	0	600	0	80	0	0	0
<i>S. waycross</i>	320	0	320	0	1,000	40	800	0	40	0	0	0
<i>S. rubislaw</i>	80	0	0	0	20	0	0	0	640	320	320	320

0 = <20

TABLE 2  
Flagellar relationships of *S. waycross*

H SERA	ANTIGENS					
	<i>S. arizonae</i> Z4Z12Z13	<i>S. cerro</i> Z4Z12Z13	<i>S. düsseldorf</i> Z4Z1	<i>S. tallahassee</i> Z4Z12	<i>S. stanleyville</i> Z4Z12	<i>S. waycross</i>
<i>S. arizonae</i>	12,800	3,200	200	100	3,200	3,200
<i>S. cerro</i>	3,200	12,800	1,600	1,600	12,800	12,800
<i>S. düsseldorf</i>	400	800	6,400	800	400	800
<i>S. tallahassee</i>	800	1,600	1,600	3,200	1,600	1,600
<i>S. waycross</i>	6,400	12,800	800	3,200	12,800	12,800

diagnostic importance. Among the salmonellae the following types of this kind are known:

*S. cerro* XVIII,  $z_4$ ,  $z_{23}$ ,  $z_{25}$

*S. düsseldorf* VI VIII,  $z_4$ ,  $z_{21}$

*S. tallahassee* VI VIII,  $z_4$ ,  $z_{21}$

*S. stanleyville* IV V XII,  $z_4$ ,  $z_{23}$

*S. arizonae* XXXIII,  $z_4$ ,  $z_{23}$ ,  $z_{27}$

The latter type, of which some strains ferment lactose belatedly, may be withdrawn from the *Salmonella* group and added to the paracolon bacilli group.

gestion that is still open for discussion. We tested these 5 types in comparison with *S waycross*. *S stanleyville*, an account of which has not yet been published, was sent to us by Dr F Kauffmann of Copenhagen. Motile H antigens were produced with these strains and tested with the corresponding antisera (except for *S stanleyville*).

Table 2 demonstrates the interrelationship of all types and the identical reactivity of *S cerro*, *S stanleyville*, and *S waycross*. *S arizona*, *S dusseldorf*, and *S tallahassee* have in addition to the  $z_4$  antigen strong individual factors which differentiate them from one another, and from the cerro-waycross-stanleyville group. These results were checked by a great many cross-absorption tests, with all sera involved, and by single or double absorptions. Scores of individual tests confirmed the conclusion that the H antigen of *S waycross* is all but identical with that of *S cerro* and *S stanleyville*. Since the designation of the *S cerro* H antigen has recently been simplified to  $z_4, z_{23}$ , this symbol is also to be given to *S waycross*, whose antigenic formula therefore reads XLI  $z_4, z_{23}$ .

#### SUMMARY

*Salmonella waycross*, a new *Salmonella* type, was isolated from the urine of a woman with chronic cystitis. It has a new *Salmonella* O antigen that also occurs in the paracolon bacillus N J 4 (Edwards), its H antigen belongs to the  $z_4$  group. The antigenic formula proposed is XLI  $z_4, z_{23}$ .

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## PHYSICAL ACTION OF SURFACE-ACTIVE CATIONS UPON BACTERIA

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A considerable amount of work has been reported on the surface-active cations, and several workers have shown that, although these compounds are highly germicidal, they are also highly bacteriostatic in relatively high dilutions. Although these compounds are highly bacteriostatic, there has been little work done to distinguish between actual germicidal activity and bacteriostatic action. Baker, Harrison, and Miller (1941) made the first attempt to separate bacteriostatic from germicidal action by the use of phospholipids. They found that the effect of the cationic compounds upon the test bacteria was seriously impaired when the bacteria were added simultaneously with the phospholipids. They were, however, unable to revive the bacteria once they had been in contact with the cationic compound. Valko and DuBois (1944) were the first workers to show the so-called reversibility of the surface-active cations. They were able by the use of "duponol PC" to neutralize in part the effect of a 1:3,000 dilution of "zephiran" upon *Staphylococcus aureus* after 10 minutes' exposure. Using *Escherichia coli* and *Eberthella typhosa*, they were able to obtain reversal up to 5 minutes. The measurement that they used for kill or survival was the absence or presence of growth in the broth subculture tubes. As mentioned by Hotchkiss (1946) this is a qualitative rather than a quantitative procedure and assumes either complete killing or no killing at all rather than the percentage surviving. Unfortunately, although such data show that neutralization has occurred, there is no information upon the degree of neutralization. The work presented in this paper further substantiates the fact that the action of the surface-active cations is reversible. The techniques used vary from those of previous workers in the method of approach and in that they are quantitative.

### EXPERIMENTAL METHODS AND RESULTS

These studies were initiated originally by the fact that when spores of *Bacillus subtilis* were exposed to surface-active cations, complete killing was obtained after short periods of exposure. The writers were firmly convinced that the bacterial spores were not susceptible to this degree to the action of these compounds and that the apparent killing was actually a bacteriostatic action. When the treated spores were subsequently treated with anionic neutralizing agents, a few spores recovered from their inhibition, but the apparent percentage of killing was still exceedingly high. It was finally decided to attempt the removal of the excess surface-active agent by centrifugation of the treated spores followed by repeated washing with sterile distilled water or physiological saline. This procedure gave

a high percentage of recovery of the spores, far greater than that obtained by neutralization by an anionic compound

In the preparation of the spore suspensions, *Bacillus subtilis* was grown on the surface of plain agar for 7 days, after which time the growth was harvested in distilled water and the resulting suspension heated to 80 C for 10 minutes for the purpose of destroying the vegetative cells and the heat-susceptible spores. This suspension was immediately cooled and preserved by storage at 10 C for subsequent use.

In the conduct of the experiments a constant amount of the spore suspension was treated in duplicate with the cationic compound giving a final concentration of the compound of 1/2,000 based upon the anhydrous material. A control containing the same amount of spores suspended in distilled water was carried along to determine as closely as possible the number of spores being treated in the various steps. These samples were centrifuged until the spores were thrown down, the supernatant was removed, sterile distilled water was added to the original volume, and an aliquot was removed for determining the bacterial count.

TABLE 1

*The reversal of bacteriostatic effect of 1/2,000 "B T C" on spores of Bacillus subtilis by centrifugation*

TREATMENT	NO RINSE	1ST RINSE	2ND RINSE	3RD RINSE
None		151,000	100,000	150,000
1	0	0	590	1,900
2	0	0	160	2,700

The suspension was then centrifuged a second and third time, the supernatant being removed and a bacterial count being made each time. Another untreated control was allowed to stand during the course of the experiment and was plated at the termination of the experiment. The data are presented in table 1. From these data it can readily be seen that there is a definite revival of the theoretically killed spores as shown by the comparison of the bacterial count of the first rinse and the treated control. Furthermore, this revival increases with the greater number of rinses.

In the foregoing experiments it was noted in every instance that the bacterial counts of the various dilutions plated failed to follow the normal pattern, e.g., the low dilution gave a lower count per plate than did the higher dilution, although the number of colonies was within the normal number of colonies (25 to 250) permissible for counting. This could only mean that the simple process of dilution and shaking was further reversing the action of the surface active cations.

Following the foregoing observation, an experiment was conducted to determine whether or not this reversal could be demonstrated by a simple dilution technique. The tubes were prepared in the same manner as in the previous experiment. From these tubes 1-ml samples were drawn at 2-minute intervals for 20 minutes, placed in 99 ml of sterile distilled water, and shaken vigorously, 70



times This diluted suspension was plated in amounts of 1 ml and 0.1 ml, and 1 ml of a 1:100 dilution A control suspension of the spores in distilled water was plated before the experiment was run, held during the course of the experiment, and then plated again at its termination to determine whether or not there had been any large change in the population during the course of the experiment due to external factors Table 2 gives a typical set of results The data show that no germicidal action occurs after the first 2 minutes of exposure The bacterial population remains practically constant for the entire 20 minutes of exposure It can be seen that in the 1:2,000 dilution of the cationic agent practically all of the spores were recovered, in the 1:1,000 dilution a kill of 50 per cent was apparent, and in the 1:500 dilution the kill was increased to approximately 90 per cent

TABLE 2

*The reversal of bacteriostatic effect of "B T C" on spores of Bacillus subtilis by dilution and shaking*

EXPOSURE TIME	NUMBER OF BACTERIA PER ML		
	Trial 1 1:2,000	Trial 2 1:1,000	Trial 3 1:500
Initial count	310,000	770,000	2,320,000
2 minutes	149,000	310,000	206,000
4 minutes	136,000	384,000	206,000
6 minutes	256,000	204,000	196,000
8 minutes	218,000	428,000	160,000
10 minutes	272,000	276,000	118,000
12 minutes	140,000	224,000	141,000
14 minutes	248,000	168,000	126,000
16 minutes	264,000	254,000	124,000
18 minutes	240,000	134,000	114,000
20 minutes	278,000	168,000	158,000

Data are presented in table 3 showing the effect of dilution by nutrient agar upon the number of colonies appearing on the plates It is interesting to note that although the 1-ml and 0.1-ml portions came from the same flask, which was the first dilution of the treated suspension, the plate count from the 0.1-ml portion in practically all cases yielded a greater number of colonies than did the 1-ml portion This would indicate that the dilution resulting from mixing the organisms in the nutrient agar tended to eliminate the bacteriostatic action This would be in addition to the reversing action of the agar itself as reported by Quisno *et al* (1946)

Much work has been reported on the electrophoretic mobilities of bacterial cells some in regard to their pathogenicity (Frampton and Hildebrand, 1944), some regarding the stage of growth of an individual organism (Moyer, 1936a), some regarding the effect of cations in general upon organisms (Moyer, 1936b), and most recently some on the effect of surface-active agents upon various bacterial cells (Dyar and Ordal, 1946) Electrophoretic mobility, using a technique

similar to that used by the foregoing workers, was used to show the removal of surface-active agents from the surface of bacteria by washing procedures.

A simple electrophoretic cell was used with a depth of 600 microns. The distance between the electrodes was 3.1 cm. To keep from encountering various currents within the electrophoretic chamber of this type, all determinations were made with the microscope focused at a distance of 100 microns below the bottom of the cover glass. This depth was chosen because it has been shown by other workers that, in a 600-micron cell, the most stationary levels are at 200 and 100 microns from the bottom of the cell. The lower level (200 microns from the bottom of the cell) was chosen for observation because the spores are relatively heavy and tend to settle toward the bottom of the cell. A constant potential of 200 volts was used for all tests, and a current of 1 milliamperes was never exceeded.

TABLE 3

*The reversal of bacteriostatic effect on spores of Bacillus subtilis by agar plating after treatment with 1:500 dilution of "B T C" as demonstrated by comparative counts obtained with 1-ml and 0.1-ml dilution agar plates*

SAMPLE NUMBER	NUMBER OF COLONIES APPEARING ON THE PLATES	
	1.0 ml	0.1 ml
1	14	206
2	33	206
3	40	196
4	45	160
5	11	118
6	63	111
7	18	126
8	63	121
9	122	114
10	66	158

In making the electrophoretic tests, the time for a single spore in the electrical field to traverse 200 microns in one direction was observed and recorded. The poles of the cell were then reversed and the time for the same spore to travel back to its original position was determined. This was done for the purpose of nullifying any currents, convection or otherwise, which may have been present at that level in the preparation. Five determinations on each preparation were made in this fashion, the 10 time intervals were averaged, and the mean was used for the calculation of the rate of speed of the spores in the electrical field.

In the tests, the samples were centrifuged and washed in the same manner used to obtain the results in table 1. Aliquots were removed after each washing and the electrophoretic mobilities determined. The concentrations of the surface-active cations were 1:1,000, 1:2,000, and 1:4,000. The mobility rate of the untreated spore suspension was determined as a control.

The data for the foregoing experiment using "roccal" as the surface-active

cation are given in table 4. Similar experiments were conducted using "B T C" and "tetiosan" as the surface-active cations, and also using as test organisms *Escherichia coli* and *Staphylococcus aureus*. The data for the last mentioned are not given because they present identically the same picture as that presented in table 4.

In the foregoing experiments no attempt was made to regulate the pH by the use of a buffering system since preliminary experiments showed only negligible differences between mobilities in the buffered and unbuffered solutions. The data show that when the organisms were treated with a 1:1,000 solution of the cationic agent, the spores changed from a negative mobility of 19 microns per second to a positive mobility of 18 microns per second. After one washing the positive charge was lost by the removal of the cationic substance from the surface of the spore and from the solution. The spore now showed a negative mobility of 16.9 microns per second. A second washing increased the negative mobility to a point greater than the negative mobility of the untreated spores.

TABLE 4

*The electrophoretic mobilities of spores of Bacillus subtilis when treated with "roccal" followed by centrifugation and washing with distilled water*

TREATMENT	MOBILITIES— $\mu$ /SEC IN 20 VOLT FIELDS		
	Dilution of cationic agent		
	1:1,000	1:2,000	1:4,000
No wash	+18.6	+16.7	+7.0
First wash	-16.9	-16.9	-20.3
Second wash	-25.5	-25.9	-19.8
None	-19.0	-19.4	-19.4

The symbols + and - represent electrical charges on spores.

These data confirm the bacteriological finding that dilution will remove the cationic compounds from the surface of the spores.

In the light of the results above, further studies were conducted on *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* using the dilution technique as a method of reversing the action of the cationic compounds. Dilutions of 1:5,000, 1:6,000, and 1:10,000 of the surface-active cations were used with an inoculum of over 10,000,000 organisms per ml. In no instance, either by shaking or centrifuging, could the organisms be recovered after an exposure of 3 minutes or more. In most cases a count of 1 or 2 colonies was found on the plates containing the 1:100 dilution of the cationic agent and organism suspension after an exposure of 1 minute. In one case a count of 1,700 per ml was obtained after the exposure of *Staphylococcus aureus* for 1 minute. This dropped to 300 per ml in 2 minutes, and then to zero in 3 minutes. This, then, would seem to indicate that vegetative cells are killed or sufficiently hampered by the action of the cation so that their recovery by either of the methods, washing or centrifuging, is not possible. Dilutions of the cationic compound greater than 1:10,000 were

not used This policy was adopted since these compounds are used in the field in dilutions of less than 1 10,000

In making the electrophoretic studies, the question arose as to whether or not clumping of the organisms would occur if the concentration of the cationic agent was such that it just neutralized the surface charge of the organisms, or if any clumping occurred spontaneously during the transition of the organism from a negative to a positive charge owing to the adsorption of the compound To determine whether or not such was the case, tests were run on *Escherichia coli*, *Eberthella typhosa*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* Twenty-four-hour broth cultures of these organisms were centrifuged to remove the broth, and were then resuspended in sterile water by vigorous shaking to assure the breaking up of all clumps that might have been formed by centrifugation These suspensions were then added to varying dilutions of three different quaternaries, namely, "roccal," "hyamine 1622," and "B T C" Glass cover slips were placed in the dilutions of cationic substances before the organism suspensions were added to facilitate the clumping of the organisms upon a glass surface, as has been reported previously After the addition of the organism suspension, the cover slips were removed, placed upon a clean slide, and examined by using dark field illumination The following results represent the three quaternaries collectively since no differences in their reactions were noted

*Pseudomonas aeruginosa*—clumping occurred up to a dilution of 1 1,000 No clumping in dilution of 1 5,000 or greater

*Escherichia coli*—clumping occurred up to a dilution of 1 2,000 No clumping in dilutions of 1 4,000 or greater, with the exception of 1 60,000, which showed no clumping immediately but did after a 24-hour period

*Eberthella typhosa* and *Staphylococcus aureus*—no clumping in any dilutions from 1 1,000 to 1 100,000

It will be recalled that in a previous portion of the paper dealing with reversal, it was noted that when working with dilutions commonly used for disinfection purposes, namely, 1 1,000 to 1 7,000, no reversal of the cationic action could be obtained with vegetative cells even though it was shown electrophoretically that the compound had been washed from the surface of the organism by shaking, which process was sufficient effectively to disperse any clumps present This, then, would seem to show that the organisms had either been killed before the clumping occurred, or, more logically, that the compound adsorbed on the surface of the organisms continues its action even though the organisms are clumped

It is of interest to note that in conducting the foregoing experiments the dark-field illumination showed those organisms that had been killed or immobilized by the cationic agent to have a much higher refractive index than normal organisms or those organisms present in the solution which had not as yet been immobilized

An application of the possible reversal by dilution and shaking was attempted by the authors while collecting data for another paper (Mallmann, Kivela, and Turney, 1946) While taking swabs of beverage glasses treated with 150 ppm of a surface-active cation, the swab bottles were shaken 30 to 50 times to the

oughly fluff the cotton of the swab immediately after the swab had been taken. In this manner it was hoped that the compound could be washed from the surface of the organism. The fact that possible bacteriostasis may occur from the compound carried over on the swab was disregarded since, from previous experiments carried on by the authors, it has been quite conclusively shown that the extremely high dilutions of the compound resulting in the swab bottles would have no effect upon the bacterial population. The results showed no difference in count between the shaken and unshaken swabs, which again demonstrates that the organisms present on the glasses were evidently killed before the action of the cationic agent was reversed.

In the light of the studies cited on electrical charges and the apparent adsorption of the cationic compounds on the surface of the organism, an attempt was made to correlate these with a possible mode of action of the cationic surface-active agents. Hotchkiss (1946) reported that the cell contents of organisms had been found in the supernatant fluid after the organisms had been acted upon by surface-active cations, and he believed that this was due to the denaturation of the cell wall, which thereby destroyed its selective permeability and allowed the cell fluids to escape into the suspending fluid. These authors thought that, in addition to the cell wall breakdown, the osmotic pressure exerted by these compounds might increase the amount of these cell fluids in the surrounding medium. Thus a study of the osmotic pressure of a surface-active cation was made, using "roccal" as a typical compound.

The results obtained are what would normally be expected judging from the molecular weight of this compound. The osmotic pressure exerted by the compound, as determined by a purely physical test, would not seem to exert an appreciable effect. However, this does not reveal the entire condition. It should be remembered that in very dilute solutions of a cationic compound enough is adsorbed on the surface of the organism to change its charge from a negative one to a positive one. This, then, would indicate that the concentration of the compound upon the surface of the organism is much greater than the concentration of the compound in the solution. The authors believe that this adsorbed material exerts an osmotic pressure comparable to its concentration on the bacterial cell. This means that an organism in a 1:1,000 dilution of a cationic compound would not be exposed to an osmotic pressure of only a 1:1,000 dilution but would be subjected to an osmotic pressure greatly in excess of that, owing to the high concentration of the compound upon the organism. This osmotic pressure would tend to draw the cell fluids through the disrupted cell wall into the surrounding fluids. This belief was further strengthened by Dyar (1947), who observed, while employing a 1:300 dilution of cetyl pyridinium chloride for a cell wall stain, that the examination of the bacterial cells in water under a cover slip showed the cytoplasm to be shrunk away from the cell wall.

It is quite probable that the surface-active agents are adsorbed on the surface of the bacterial cells in sufficient concentration to interfere with the osmotic balance of the organism and its surrounding medium, and in this manner may prevent the intake of nutrients. This would help to explain why these

compounds are so highly bacteriostatic in high dilutions, and why the bacteriostatic effect can be removed by dilution and vigorous shaking or by the use of neutralizing agents. Further, it would explain partially why reversal can be obtained using spores, since their cell wall is much thicker than that of vegetative cells and consequently more resistant to chemical changes as well as osmotic effects.

#### SUMMARY

The bacteriostatic effect of surface-active cations on bacterial spores can be reversed by dilution and shaking in distilled water or physiological saline solution.

The reversal of the bacteriostatic effect of surface-active cations by the removal of the cations adsorbed on the bacterial spores by dilution was proved by demonstrating that negative mobilities of the spores were restored by washing the cells with distilled water.

The high osmotic pressure exerted on the bacterial cell by the adsorbed surface-active cation may explain, in part, the destruction of vegetative cells by the discharge of cell fluids into the suspending solution.

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# STUDIES ON POLYMYXIN THE PRODUCTION OF FERMENTATION LIQUOR

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The antibiotic polymyxin occurs in the cell-free fermentation liquor of a strain of *Bacillus polymyxa* isolated in these laboratories. The origin, production, isolation, and biological activity of this substance have been briefly described in a previous publication (Stansly, Shepherd, and White, 1947).

The present communication describes in detail a method for the routine production of high potency fermentation liquor on a large laboratory scale. The data presented indicate certain nutritional and environmental factors that were found to have an important bearing on production.

## METHODS

Unless otherwise stated, experiments were conducted at 25 C in 500-ml Erlenmeyer flasks, 100 ml of medium per flask. Media were prepared in distilled water, adjusted to pH 7 with NaOH, and sterilized by autoclaving for 15 minutes at 121 C. The flasks were inoculated with 0.1 ml of a 24-hour "trypticase soy"<sup>1</sup> broth culture of *Bacillus polymyxa* incubated at 37 C.

In all instances, experimental flasks were in triplicate and an average potency was obtained from individual assays of the centrifuged fermentation liquor of each flask. Activity was determined either by the agar streak, serial dilution method (Waksman and Reilly, 1945) using *Escherichia coli* (MacLeod strain), and expressed as a dilution, or by the more accurate agar diffusion method (Stansly and Schlosser, 1947) and expressed as units per ml. An assay titer of 1:128 is approximately equivalent to 128 units per ml.

## EXPERIMENTAL RESULTS

**Nutritional factors** Table 1 summarizes the effect of some simple and complex nitrogen sources, either alone or in combination, in a basal medium consisting of 1 per cent glucose, 0.2 per cent  $\text{KH}_2\text{PO}_4$ , 0.05 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001 per cent  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.005 per cent NaCl. Incubation was at 30 C. It is apparent that the basal medium plus 1 per cent  $(\text{NH}_4)_2\text{SO}_4$  and 0.5 per cent yeast extract (medium 5) gave the highest potency.

Preliminary experiments to determine the optimum concentrations of some of the constituents of medium 5 indicated that production was unaffected when the yeast extract was increased from 0.5 to 1 per cent. On the other hand, the potency of the liquor was decreased by one-half when the yeast extract was decreased to 0.125 per cent. An increase in glucose concentration from 1 to 2

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per cent did not affect the potency obtained in medium 5, whereas an increase of ammonium sulfate from 1 to 2 per cent appeared to yield a more active liquor

Based on these findings, a medium consisting of the basal ingredients plus 0.5 per cent yeast extract (Difco) and 2 per cent  $(\text{NH}_4)_2\text{SO}_4$  became the standard medium for further experiments and for routine production

It is interesting to note that the production of activity was not related solely to the amount of growth. This can be seen in table 1 by comparing the potency and growth obtained in media 1, 4, and 5. The lack of strict parallelism of growth and potency has been observed with respect to other factors affecting production of polymyxin.

When tested in a concentration of 1 per cent, cerelose,<sup>2</sup> sucrose, and soluble

TABLE 1  
*Effect of some sources of nitrogen on production of polymyxin*

MEDIUM NO	BASAL* PLUS		GROWTH†	ACTIVITY‡
1	Tryptone	1 per cent	++	0
2	Casein hydrolyzate	1 per cent	0	0
3	$(\text{NH}_4)_2\text{SO}_4$	1 per cent	0	0
4	Tryptone Yeast extract	1 per cent 0.5 per cent	+++	1.8
5	$(\text{NH}_4)_2\text{SO}_4$ Yeast extract	1 per cent 0.5 per cent	++	1.256

\* One per cent glucose, 0.2 per cent  $\text{KH}_2\text{PO}_4$ , 0.05 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001 per cent  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.005 per cent  $\text{NaCl}$

† The number of + signs designates the relative amount of growth observed by visual inspection after 2 days of incubation, 0 denotes absence of visible growth

‡ Activity determined by the serial dilution, agar streak method, after 3 days of incubation

starch (Merck) were approximately equivalent to glucose, giving after 4 days of incubation 197, 213, and 167 units per ml, respectively, compared to 210 for glucose. On the other hand, lactose seemed somewhat less favorable, the average potency being 116 units per ml.

Brewer's yeast, fish solubles, distillers' solubles, corn steep liquor, and soybean oil meal<sup>3</sup> were tested in a concentration of 1 per cent (dry weight) as substitutes for yeast extract in the standard medium. The resulting potencies after 4 days of incubation were, respectively, 31, 38, 45, 88, and 132 units per ml compared to 132 units per ml for 0.5 per cent yeast extract.

<sup>2</sup> Commerical hydrated glucose

<sup>3</sup> Brewers' yeast, Spert, type I, fish solubles, Gorton Pew Company, distillers' solubles ("stimufav"), Hiram Walker and Sons, corn steep liquor, Corn Products Refining Company, soy bean oil meal, Swift and Company. We wish to thank Dr. I. M. Prucha of Lederle Laboratories for making these products available.



Tap water gave results similar to distilled water, and technical ammonium sulfate was found to be equivalent to cp grade. Equivalent potencies were obtained whether the glucose was autoclaved with the balance of the medium or sterilized separately and added to an autoclaved solution of the other ingredients. The former procedure, however, resulted in a dark beer from which the active principle was somewhat more difficult to obtain as a colorless salt.

From the standpoint of large-scale fermentation it was of interest to determine the effect of certain construction metals on the production of polymyxin. For this purpose, 5 square centimeters of metal surface per 100 ml of medium were autoclaved with the medium and retained in the flask for the fermentation period. It was observed that the addition of stainless steel consistently resulted in potencies equal to or higher than the controls. On the other hand, the addition of black iron and construction steel consistently resulted in potencies somewhat lower. Typical results were 107, 94, and 139 units per ml, respectively, for black iron, construction steel, and stainless steel, compared to 142 for the control.

Unlike the case of bacillin (Foster and Woodruff, 1946), the addition of manganese (10 ppm as  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ) to the standard medium had no favorable effect on the production of polymyxin and, in fact, appeared to depress it.

*Incubation temperature* Fermentation at 20, 25, and 30 C resulted in equivalent potencies when assayed after 3 days. Although growth was very good at 37 C, the resulting potency was decreased roughly 15-fold.

*Incubation period* At 25 C, using the customary inoculum, maximum potency was reached at about the third day and remained at approximately a constant level for a period of at least 28 days. High potency has been observed to occur before appreciable sporulation took place, sporulation was usually complete on the fifth day.

*Aeration in deep fermentation* It was observed that if the volume of medium was increased in the 500-ml Erlenmeyer flask, the potency was correspondingly less. Thus, 100, 200 and 300 ml per flask gave, respectively, a titer of 1/128, 1/64, and 1/32 after 7 days of incubation at 30 C. These results suggested the necessity for forced aeration in deep fermentation. This was shown to be the case in an experiment in which 12-liter round-bottom flasks containing 8 liters of medium were aerated at the rates of 0, 1, 2, 4, 8, 16, and 64 liters of air per hour. After 4 days of incubation, the potencies were, respectively, 10, 77, 96, 138, 127, 140, and 153 units per ml. It is apparent that, under these conditions, aeration at the rate of at least 4 liters per hour was essential for maximum production.

It might be supposed that the effectiveness of aeration was due to supplying increased amounts of oxygen to the metabolizing organisms. An alternative explanation suggested by Adams and Leslie (1946) for the production of 2,3-butanediol by *B. polymyxa* was that the surface volume relationships, which are similar to those required for polymyxin production, reflect the need to eliminate metabolic gases (e.g.,  $\text{CO}_2$ ). That this may also be the case with polymyxin was demonstrated by the fact that equally good growth and production of polymyxin were obtained by aerating with nitrogen (99.7 per cent pure), at 64 liters per hour, as with air at the same rate.

*Control of foaming* With forced aeration, foaming was a serious problem. Of a variety of substances tested, a solution of 1 per cent octadecanol in mineral oil (U S P) appeared to be the most effective agent in controlling this difficulty. Its effectiveness was confirmed in routine production. In the amounts used no particular effect was observed either on the growth of the organisms or on the production of polymyxin.

*Changes in pH during fermentation* Routine pH determinations indicated that during large-scale production of polymyxin a small but characteristic fluctuation of pH took place. If production failed, however, the pH changes were characteristically different. During successful production, the pH would decrease from an initial value of about 7 to about 6 by the third day and then increase to a value close to neutrality by the fifth day. Such a beer had a pleasant, yeasty odor. If the pH decreased to about 5, however, little or no production of polymyxin took place. Such a beer usually had a sharp, disagreeable odor. Contaminating organisms could not be demonstrated in these unproductive fermentations.

#### *Description of Large-Scale Laboratory Production of Polymyxin*

One hundred and eighty liters per week of fermentation liquor have been routinely produced in twelve 22-liter, round-bottom pyrex flasks containing 15 liters per flask. As a safety measure and as a convenience in handling, the flasks were enclosed in protective holders fabricated from galvanized iron and cushioned with rubber tubing.

To each flask were added 75 ml of 10 per cent NaCl, 600 ml of 50 per cent  $(\text{NH}_4)_2\text{SO}_4$ , 300 ml of 10 per cent  $\text{KH}_2\text{PO}_4$ , 75 ml of 10 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 ml of 1 per cent  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 750 ml of 10 per cent (Difco) yeast extract, and 12,650 ml of water. The pH was adjusted to 7.9 to 8.0 (requiring about 23 ml of saturated NaOH). After being stoppered with cotton wool and gauze, the solution was sterilized by autoclaving for 1 hour at 15 pounds' steam pressure (approximately 121 C).

After the solution reached room temperature, the following were added: (a) 600 ml of a 25 per cent solution of glucose in distilled water (sterilized for 20 minutes at 15 pounds), (b) 15 ml of a 24- to 48-hour culture of *Bacillus polymyxa* (from a small flask of the standard medium, 100 ml in a 500-ml Erlenmeyer, incubated at 25 C), and (c) 15 ml of a 1 per cent solution of octadecanol in mineral oil (sterilized by heating in an oven for 1.5 hours at 160 C).

The cotton plug of each flask was replaced with an aeration assembly (described below) sterilized in a specially constructed galvanized iron can. After being connected to the compressed air line and the flowmeter, the flasks were aerated at the rate of 64 liters per hour for 5 days at 25 C.

The initial pH of the medium in lots 14 to 28 varied from 6.9 to 7.6 (avg 7.3). On the third day of incubation the pH was 6.0 to 6.6 (avg 6.3) and the potency 159 to 286 (avg 203) units per ml. On the fifth day, the pH was 6.6 to 7.0 (avg 6.8) and the potency 163 to 358 (avg 216) units per ml.

*The aeration assembly* The aeration assembly consisted of a 12-inch stain-

less steel inlet tube (diameter 1.36 inches) packed with glass wool (Corning no 800) and soldered to a 14-inch stainless steel tube (diameter 8 mm) by means of a steel disk. The tubing of the smaller diameter was pushed through a no. 14 rubber stopper until it reached within a few inches of the bottom of the flask. At this end was attached, by means of a small section of rubber tubing, a cylindrical sintered glass dispersion tube (Corning no. 12c).

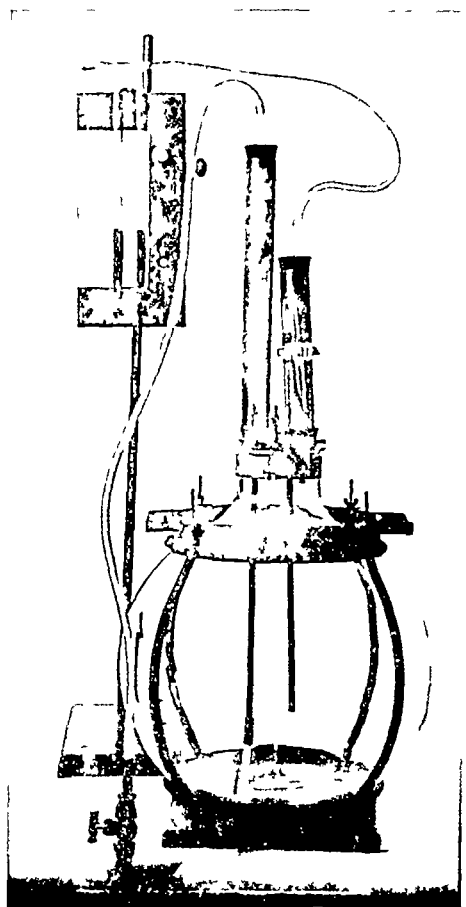


FIG. 1. FERMENTATION UNIT FOR PRODUCTION OF POLYMYXIN.

The air outlet consisted of a 7-inch stainless steel tube (diameter 1.36 inches) packed with glass wool, soldered as described above to a short length (2.5 inches) of 8-mm stainless steel tubing, and inserted by means of this into the same no. 14 stopper. The air inlet tube was connected to the air line by means of a no. 7 rubber stopper and rubber tubing, and the air outlet was connected to the flowmeter in the same manner.

The sampling device consisted of a 16-inch length of stainless steel tubing (8 mm in diameter) pushed through the large rubber stopper and reaching well

into the interior of the flask. To the outer end was attached, by means of a short piece of rubber tubing, a bent (180-degree) two-way pyrex stopcock lubricated with silicone grease. The outer end of the stopcock was drawn out to provide the sampling nipple.

Aseptic sampling was achieved in the following way. The air outlet tube was disconnected from the flowmeter and replaced with a solid rubber stopper. After a minute, sufficient pressure was built up in the flask to force liquid up through the steel sampling tube and out the stopcock. The first 10 ml were discarded. Backflow, and contamination, were prevented by shutting off the flow while the solid rubber stopper was still in place.

The aeration assembly was secured to the flask by a galvanized iron band across the diameter of the no. 14 stopper. The band was joined at each end to an iron collar around the neck of the flask, permitting tightening of the stopper.

*The flowmeter.* The flowmeter consisted of an open "U" tube with a glass "T" attached to one end. One arm of the "T" was connected to the air outlet tube and the other to a short length of capillary tubing. The "U" tube was partially filled with either mineral oil or dibutyl phthalate colored with Sudan IV. Each flask was connected to an individual flowmeter that was previously calibrated with a wet-test gas meter.

Figure 1 illustrates the complete fermentation assembly.

#### SUMMARY

Certain nutritional and environmental factors influencing the production of polymyxin by *Bacillus polymyxa* are described. The requirements for large-scale laboratory production and a complete assembly suitable for this purpose are described in detail.

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## NOTE

### CHROMOGENIC BACTERIA RELATED TO BACTERIUM GLOBIFORME<sup>1</sup>

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Recently Conn and Dimmick (J Bact, **54**, 291) in considering soil bacteria similar in morphology to *Mycobacterium* and *Corynebacterium* have proposed a new genus, *Arthrobacter*, for the inclusion of soil types which, though related to *Corynebacterium*, were regarded as having characteristics sufficiently different from those of the type species (the diphtheria organism) to warrant generic distinction from the latter. The accommodation of bacteria of the *Bacterium globiforme* group in a separate genus has much to commend it, by avoiding undue extension of the commonly held definition of *Corynebacterium* while providing for an important group of related forms. More difference of opinion might be ex-

CROP	TOTAL BACTERIA (PERCENTAGE CHROMOGENIC)		B GLOBIFORME GROUP ONLY (PERCENT AGE CHROMOGENIC)	
	Control soil	Rhizosphere	Control soil	Rhizosphere
Red clover	12.1	23.7	0.0	66.7
Mangels	12.1	29.2	0.0	12.5
Oats	12.1	22.9	0.0	50.0
Tobacco	1.7	27.8	0.0	68.7
Corn	3.8	33.3	0.0	0.0
Flax	7.7	27.1	0.0	11.8

pected to arise out of the problem of specific distinction within the proposed genus *Arthrobacter*, and much wider surveys than have so far been reported appear to be required before differences within the group can be rightly assessed, considering the pleomorphism and "unstable" physiology which seem to characterize many of the indigenous soil types.

The classification of the chromogenic (yellow) forms related to *Bacterium globiforme* appears to merit more attention than has been generally given. Such forms appear to have been encountered much less frequently than nonchromogenic types, and indeed quite rarely by some investigators, though there is reason to believe that they are common in soil. They appear, however, to be largely associated with the growing plant. Hitherto unreported data, obtained during a study of the influence of plant growth on the character of the soil bacterial flora (Lochhead Can J Research, **18**, 42), have shown that the percentage of chromo-

<sup>1</sup> Contribution No. 254 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.

genic types of the *Bacterium globiforme* group (as determined by nonselective isolation procedures) was markedly increased in the rhizosphere (see table).

In the experiments in question no chromogenic cultures of the *Bacterium globiforme* group were obtained from the control soils, whereas 12.3 per cent of all strains of this group isolated from soil adhering to the roots were yellow forms. In the rhizosphere, there is, moreover, a preferential stimulation of chromogenic types in general.

# FACTORS INFLUENCING THE PRODUCTION OF LIPASE BY *MYCOTORULA LIPOLYTICA*<sup>1</sup>

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Pasteurization of milk is known to destroy milk lipase that is essential in the normal ripening of blue or Roquefort type cheese. This study was undertaken to determine whether a microbial lipase suitable for possible use in the manufacture of blue cheese from pasteurized milk could be produced and to furnish information relative to the factors that influence lipase production by *Mycotorula lipolytica*.

Among the many lipolytic microorganisms reported in the literature, only four of those known to be highly lipolytic were considered in this study, namely, *Achromobacter lipolyticum*, *Alcaligenes lipolyticus*, *Pseudomonas fragi*, and *M lipolytica*. *A lipolyticum* was identified by Huss (1908) as causing rancidity in milk, and was studied further by Collins (1933), Long and Hammer (1938, 1939), and Fouts (1939), all of whom reported that the organism was highly lipolytic. Long (1936) characterized *Alcaligenes lipolyticus*, previously known as *Bacillus abortus* var *lipolyticus* and *Bacterium lipolyticus* (Evans 1917, 1918), and Long and Hammer (1938, 1939) showed its pronounced lipolytic effect upon stored butter. *P fragi* was named and characterized by Hussong (1932), and Long (1936) and Long and Hammer (1938, 1939) demonstrated further the lipolytic activity of this organism. Harrison (1927) characterized *M lipolytica* as capable of hydrolyzing fat. Further studies on this microorganism were made by Long (1936), Long and Hammer (1938, 1939), and Fouts (1939), all of whom were able to demonstrate considerable lipase activity in milk, cream, and butter.

Relatively little information concerning the factors that influence lipase production by microorganisms was found in the literature. Weisbrodt (1927) and Naylor, Smith, and Collins (1930) were able to increase lipase production of *Penicillium roqueforti* by substituting ammonium chloride for sodium nitrate in Czapek's medium as modified by Dow, and by adjusting the reaction to pH 4.5. Thibodeau and Macy (1942) observed that sugar retarded the production of lipase by *P roqueforti*, but the presence of both agar and organic nitrogenous compounds increased lipase production.

After preliminary studies of the production of lipase by *Achromobacter lipolyticum*, *Alcaligenes lipolyticus*, *P fragi*, and *M lipolytica*, the last organism was selected for an investigation of the factors that influence lipase production.

## METHODS

The nutrient broth used as the basal medium for lipase production in most of the studies contained 5 g Difco peptone, 3 g beef extract, 0.5 g glucose, 500 ml

<sup>1</sup> Journal Paper J-1482 of the Iowa Agricultural Experiment Station, Project 895

distilled water, 230 ml 0.6 M  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , and 270 ml 0.3 M  $\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ , and the reaction was adjusted to pH 4.5. One drop of 24- to 48-hour culture in litmus milk was added to 200 ml of sterile basal medium in a 500-ml Erlenmeyer flask stoppered with a cotton plug. Cultures were grown at 30 C unless it is stated otherwise. In the earlier experiments the cultures for lipase production were shaken twice daily, but in later trials the shaking was omitted, except at the time of sampling.

Tryptone glucose extract agar (American Public Health Association, 1911) was used for pouring plates for the enumeration of organisms. All plates were counted after being held for 48 hours at 30 C. Methylene blue or gentian violet stain was used in preparing slides for the microscopic examination of cells.

For the quantitative determination of lipase activity, the butterfat-agar emulsion used contained 5 g butterfat, 0.25 g agar, 80 ml distilled water, 10.5 ml 0.6 M  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 4.5 ml 0.3 M  $\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ , and formaldehyde (1:1,500), and the reaction was adjusted to pH 6.5. The water, buffer, and agar were mixed, heated to boiling, cooled until the agar had solidified, and tempered overnight at 45 C. Butter was heated in boiling water and the butterfat decanted and tempered at 45 C overnight. The butterfat and formaldehyde were added to the agar, and the mixture was shaken well and homogenized twice with a hand homogenizer so as to obtain a stable emulsion. The emulsion was placed in clean 6-oz screw-cap bottles in 50-ml quantities, this being the amount that was used for each determination of lipase activity. Inoculations for lipase determination were made at the rate of 2 per cent (1 ml) of culture or supernatant, as the case might be, followed by a 48-hour incubation period at 37 C, with shaking twice daily. The control run on every active culture was identical in all respects to the active sample, except that the culture or supernatant that was being tested for lipase activity had been heated to boiling for 15 minutes in order to destroy the lipase enzyme.

Titration was made in duplicate on 10-g portions of the emulsions to which the heated and unheated material had been added. To each weighed sample were added 50 ml of a 50:50 mixture of neutral 95 per cent ethyl alcohol and diethyl ether. The free fatty acids were titrated with N/20 potassium hydroxide in absolute methyl alcohol, according to the method of Bird (1946), using phenolphthalein as indicator. The difference in titration values between the active sample and the control was regarded as due to lipase activity. Lipase activity was calculated and expressed in acid degrees, which are defined as the number of milliliters of N/1 sodium hydroxide required to neutralize the free fatty acids in 100 g of fat (Association of Official Agricultural Chemists, 1910).

## RESULTS

Preliminary trials with five different growth media showed that nutrient broth containing 0.05 per cent glucose was most suitable for lipase production by *M. lipolytica*. Reconstituted skim milk or whole homogenized milk, as well as two partially defined synthetic media, proved unsatisfactory. Further trials with nutrient broth, both with and without 0.05 M disodium phosphate plus



monosodium phosphate added and adjusted with N/1 hydrochloric acid or N/1 sodium hydroxide to reactions ranging from pH 3.5 to 8.3, showed highest lipase production in the unbuffered medium when the initial reaction was in the range from pH 4.5 to 6.5, and in the buffered medium when the initial reaction was either pH 4.5 or 5.5. Over a growth period of 136 hours a shifting of the pH toward the alkaline side as a result of growth was observed in all media except those initially at pH 8.3.

In a study designed to determine the suitability of different buffers as well as different concentrations of the buffers in question, two trials were conducted. The results of one representative trial are shown in table 1. The results of both

TABLE 1

*The influences of various buffer pairs at different concentrations and pH levels upon lipase production and growth of M. lipolytica*

BUFFER PAIRS	BUFFER CON- CENTRATION (MOLAL)	pH OF MEDIUM		LIPASE ACTIVITY† PER ML OF CULTURE AFTER GROWTH PERIODS OF				COUNT PER ML (IN MILLIONS) AFTER GROWTH PERIODS OF			
		Initial	Final*	48 hr	72 hr	96 hr	144 hr	48 hr	72 hr	96 hr	144 hr
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O + KH <sub>2</sub> PO <sub>4</sub>	0.225	7.5	7.7	32	30	13	15	12	14	11	13
	0.075	7.5	7.9	31	23	16	11	15	18	23	14
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O + C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> H <sub>3</sub> O	0.225	7.5	8.0	21	26	50	22	1.1	35	10	12
	0.075	7.5	8.0	31	20	24	8	13	17	21	8.4
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O + KH <sub>2</sub> PO <sub>4</sub>	0.225	5.5	6.0	50	50	44	47	13	17	9	8.6
	0.075	5.5	6.4	42	45	32	34	24	14	9.5	7.1
Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O + C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> H <sub>3</sub> O	0.225	5.5	6.2	40	45	40	36	10	12	10	6.6
	0.075	5.5	6.4	52	44	42	35	31	11	6.7	6.9
C <sub>2</sub> H <sub>3</sub> OONa 3H <sub>2</sub> O + C <sub>2</sub> H <sub>4</sub> OOH	0.225	5.5	8.4	3	14	12	4	7	12	9.5	3.7
	0.075	5.5	8.8	31	32	23	19	26	17	9.3	13

\* At 144 hr

† Expressed as acid degrees

trials showed that the cultures grown in the presence of sodium acetate and acetic acid produced the least lipase, especially at the buffer concentration of 0.225 M. The phosphate and the phosphate-citrate both proved satisfactory and were more suitable for lipase production at pH 5.5 than at pH 7.5. The higher level of concentration of these two buffers proved more desirable, apparently since less shifting of reaction toward the alkaline side occurred over the 144-hour growth period. Cultures grown at the lower buffer concentrations showed more rapid growth than those grown at the higher concentrations, but they shifted in reaction more rapidly. Based on the results of both trials, the disodium phosphate plus citric acid buffer at a concentration of 0.225 M was selected for additional studies.

In further studies on the effect of pH upon lipase production by *M lipolytica* made after the useful influence of 0.225 M phosphate-citrate buffer upon lipase

TABLE 2

*Additional studies of the influence of pH upon lipase production, growth, and shape of cells of M lipolytica*

(Results of two trials)

PH OF MEDIUM	LIPASE ACTIVITY* PER ML OF CULTURE		COUNT PER . $\mu$ L (IN MILLIONS)		PREDOMINANT SHAPE OF CELLS†
	Trial 1	Trial 2	Trial 1	Trial 2	
48-hour cultures					
3.5	3.0	2.0	0.8	0.7	Short oval
4.5	28.0	17.0	2.8	10.0	Long oval, slender
5.5	33.0	20.0	2.1	17.5	Long oval, slender
6.5	25.0	8.0	3.0	6.2	Long, short oval
7.5	23.0	7.0	3.6	2.3	Long, short oval
8.5	10.0	0.0	1.3	1.3	Short oval
72-hour cultures					
3.5	1.0	7.0	1.6	1.3	Short oval
4.5	40.5	44.0	3.7	33.0	Long oval, slender
5.5	35.0	29.0	3.5	14.3	Long oval, slender
6.5	34.0	18.0	2.2	13.4	Short oval, long slender
7.5	27.0	14.0	5.5	6.8	Short, long oval
8.5	15.0	6.0	3.1	1.8	Short oval
96 hour cultures					
3.5	12.0	7.0	4.6	2.4	Short oval
4.5	64.0	44.0	5.0	18.0	Long slender, filaments
5.5	43.0	23.0	3.5	15.0	Short slender, filaments
6.5	33.0	21.0	2.1	17.0	Long, short oval
7.5	24.0	18.0	5.7	19.0	Short, long oval
8.5	21.0	6.0	3.5	4.4	Short, long oval
144-hour cultures					
3.5	12.0	16.0	3.9	9.4	Short oval
4.5	52.0	43.0	5.5	20.0	Long oval, slender
5.5	34.0	24.0	3.8	7.7	Long, short oval
6.5	17.0	19.0	2.1	5.0	Short oval
7.5	15.0	7.0	6.3	9.6	Short oval
8.5	10.0	5.0	3.0	5.9	Short oval

\* Expressed as acid degrees

† Examinations of first trial only

production had been demonstrated, the results as shown in table 2 were obtained

The highest acid degree values were obtained at pH 4.5 when the culture had grown for 72 hours or longer, with pH 5.5 proving best after growth for 18 hours

and being somewhat less satisfactory when longer growth periods were employed. Further trials usually were conducted at pH 4.5, with a few at pH 5.5.

The influence of glucose concentration was demonstrated in two trials using buffered nutrient broth at pH 4.5 with 0.0, 0.01, 0.05, 0.25, and 1.0 per cent glucose added to different lots. Examinations were made for lipase production,

TABLE 3

*The influence of glucose concentration upon lipase production, cell count, and shape of cells of *M. lipolytica**

PER CENT GLUCOSE	LIPASE ACTIVITY* PER ML OF CULTURE	pH OF MEDIUM†	COUNT PER ML (IN MILLIONS)	PREDOMINANT SHAPE OF CELLS
48-hour cultures				
0.00	16	4.7	5.1	Long slender
0.01	14	4.7	4.8	Long slender
0.05	14	4.7	5.4	Long slender
0.25	14	4.7	8.7	Long slender, short oval
1.00	5	4.7	7.9	Short oval
72-hour cultures				
0.00	30	4.8	3.5	Long slender
0.01	23	4.8	3.3	Long slender
0.05	23	4.8	3.5	Long slender
0.25	23	4.7	8.7	Short oval
1.00	12	4.6	8.0	Short oval
96-hour cultures				
0.00	24	4.9	4.2	Long slender
0.01	24	4.9	4.7	Long, short oval
0.05	24	4.9	5.1	Long, short oval
0.25	19	4.7	7.2	Long, short oval
1.00	16	4.6	6.5	Long oval
144-hour cultures				
0.00	22	5.2	5.2	Long oval
0.01	21	5.2	6.5	Long, short oval
0.05	22	5.2	6.9	Long, short oval
0.25	19	5.0	8.5	Long, short oval
1.00	17	4.8	7.4	Long oval

\* Expressed as acid degrees

† At time enzyme concentration was determined

cell count, and the morphological characteristics of cells after growth for 48, 72, 96, and 144 hours. The results of one representative trial are shown in table 3. The absence of glucose or the presence of low concentrations resulted in earlier and more rapid lipase production. Concentrations of 0.25 and 1.00 per cent glucose retarded the production of lipase, the effect being more pronounced at

the higher concentration of sugar. The presence of glucose up to 0.05 per cent was more satisfactory than greater concentrations for highest lipase production over the period studied.

The effect of temperature upon lipase production was studied using nutrient broth containing 0.05 per cent glucose and buffered at pH 4.5 with growth temperatures of 21, 25, 30, and 36 C. Examinations for lipase production and the population of cells were made after growth periods of 48, 72, 96, and

TABLE 4

*The influence of different growth temperatures on the production of lipase and the growth of M. lipolytica*

GROWTH TEMPERATURE (C)	LIPASE ACTIVITY* PER ML OF CULTURE		COUNT PER ML (IN MILLIONS)	
	Trial 1 (pH 4.5)	Trial 2 (pH 5.5)	Trial 1 (pH 4.5)	Trial 2 (pH 5.5)
48 hour cultures				
21	1.0	4.0	1.2	0.9
25	18.0	15.5	4.4	1.9
30	29.0	39.0	2.8	1.5
36	1.0	1.0	0.2	0.2
72 hour cultures				
21	14.0	9.0	3.4	1.9
25	18.5	25.0	5.1	4.7
30	39.5	34.0	4.1	1.4
36	5.0	1.0	0.3	0.01
96-hour cultures				
21	30.5	17.0	3.3	1.6
25	17.0	25.0	6.7	6.6
30	44.0	46.0	4.1	0.9
36	11.0	1.0	0.3	0.01
144-hour cultures				
21	38.0	24.0	5.6	1.5
25	32.5	33.0	12.0	7.7
30	46.5	54.0	1.8	1.4
36	3.5	0.0	0.4	0.05

\* Expressed as acid degrees

**144 hours** A second trial was run in the same manner, except that the medium was adjusted to pH 5.5. The results of the two trials are shown in table 1. The results of both trials showed 30 C to be the optimum for lipase production. Growth was most rapid and total population the greatest in cultures held at 25 C, and this temperature was second only to 30 C in favoring lipase production. An incubation temperature of 36 C definitely was inhibitory to both growth and lipase production, whereas lipase production was somewhat retarded at

21 C but eventually reached levels that compared favorably with the optimum

A series of trials in which the yeast was grown under different oxidation-reduction conditions was conducted. In the first trial, 12 ml of a 10 per cent solution of methylene blue was added to 1,200 ml of nutrient broth, buffered at pH 5.5. The medium was divided into five equal portions, inoculated, and the portions were treated variously. The control culture was shaken twice daily. Bacteria-free filtered air was bubbled through the second culture at a slow rate from a sintered glass gas-washing head. To each of three additional

TABLE 5

*The influence of oxidation-reduction conditions upon lipase production and growth of M. lipolytica*

VARIABLE FACTOR	LIPASE ACTIVITY* PER ML OF CULTURE	COUNT PER ML (IN MILLIONS)	COLOR OF MEDIUM†	PREDOMINANT SHAPE OF CELLS
48-hour cultures				
Control	8.0	3.5	Blue	Long oval
Aeration	4.0	14.0	Blue	Short oval
Ascorbic acid	3.5	2.7	Yellowish green	Short oval
Cysteine	14.0	5.8	Blue	Long oval
Thioglycolic acid	11.0	6.5	Yellow	Long oval
96-hour cultures				
Control	18.0	9.3	Blue	Long oval
Aeration	6.0	28.0	Blue	Short oval
Ascorbic acid	22.5	14.0	Greenish yellow	Short, long slender
Cysteine	28.0	11.0	Green	Long oval
Thioglycolic acid	24.0	15.0	Yellow	Long oval
144-hour cultures				
Control	32.5	8.2	Blue	Long oval, slender
Aeration	5.0	31.0	Blue	Short oval
Ascorbic acid	28.0	11.0	Greenish yellow	Short, long slender
Cysteine	35.0	11.0	Green	Long oval
Thioglycolic acid	31.5	14.0	Light green	Long oval

\* Expressed as acid degrees

† Used as indicator of oxidation-reduction conditions

lots, ascorbic acid, cysteine, and thioglycolic acid, respectively, were added to the medium to give a concentration of 0.02 per cent. These cultures were shaken twice daily. Examinations for lipase production, cell count, color changes of methylene blue, and shape of cells were made after growth for 48, 96, and 144 hours. The results are shown in table 5. Aeration definitely restricted lipase production, but the addition of cysteine increased lipase production slightly above that of the control. The beneficial effects of ascorbic acid and thioglycolic acid were doubtful.

TABLE 6

*The influence of shaking cultures upon the production of lipase, growth, and shape of cells of M. lipolytica*

VARIABLE FACTORS	LIPASE ACTIVITY* PER ML OF CULTURE		COUNT PER ML OF CULTURE (IN MILLIONS)		PREDOMINANT SHAPE OF CELLS	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
48-hour cultures						
a Shaken every 12 hours	15	21.5	6.8	13	Short, long oval	Long oval
b Shaken every 48 hours	66	38	30	28	Long slender	Long slender
96 hour cultures						
a Shaken every 12 hours	34	26	11	11	Long oval, slender	Long, short slender
b Shaken every 48 hours	35.5	22	30	28	Long slender	Long, short slender
144-hour cultures						
a Shaken every 12 hours	43	21	9	6.3	Long oval, slender	Long, short slender
b Shaken every 48 hours	22	14	38	47	Long slender	Long, short oval

\* Expressed as acid degrees

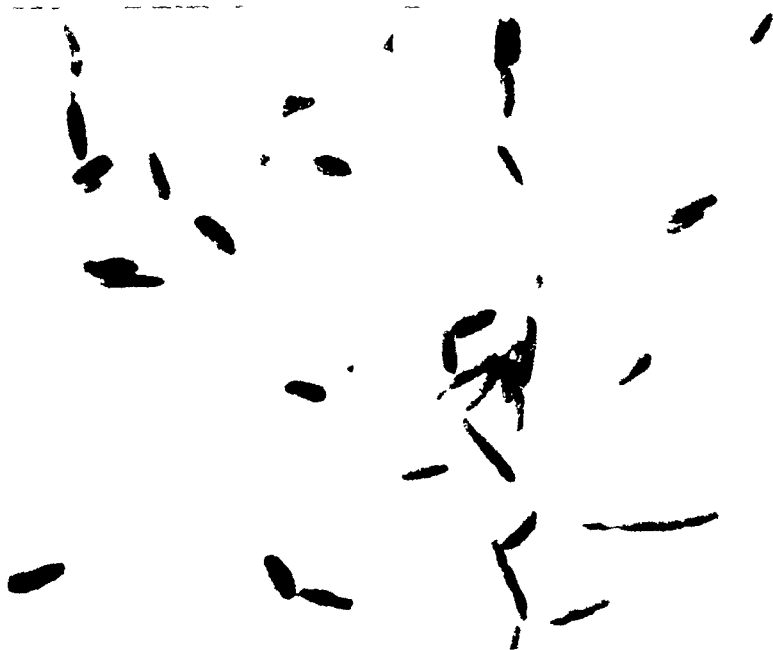


FIG 1 MYCOTORULA LIPOLYTICA, 48-HOUR CULTURE IN NUTRIENT BROTH. Culture a (at left), shaken every 12 hours, contains mostly long, oval cells. Lipase activity equivalent to 15 acid degrees per ml of supernatant culture medium. Culture b (at right), shaken every 48 hours, contains mostly long slender cells (magnified 1,125 $\times$ ). Lipase activity equivalent to 66 acid degrees per ml of supernatant culture medium.

In a further study on the influence of aeration upon lipase production, two trials were made with nutrient broth buffered at pH 4.5. Culture *a* was shaken every 12 hours whereas culture *b* was shaken every 48 hours. Examinations for lipase production, population, and shape of cells were made after 48, 96, and 144 hours of growth. The results are shown in table 6. Greater lipase activity, as measured by acid degree values, was obtained from culture *b*, which was shaken only every 48 hours, than from *a*, shaken every 12 hours. Acid degree values at 144 hours were in favor of the cultures shaken every 12 hours, but the best values at this time were lower than those at 48 hours. The cell population was much greater, even in the earlier stages, for the cultures shaken less frequently.

Throughout the study high lipase activity generally was associated with the presence of long oval or slender cells and commonly a somewhat smaller population of organisms. Rapid growth, resulting in high cell counts under conditions such as the presence of 1 per cent glucose, optimum growth temperature (25 C), and aeration, gave a predominance of short oval cells and the accumulation of little lipase. The data in tables 2, 3, 5, and 6 show this relationship. Figure 1*a* shows typical short and long oval cells from a preparation the lipase activity of which was equivalent to 15 acid degrees (culture *a*, 48 hours, table 6), and figure 1*b* shows the long, slender cells from a preparation in the same series having a lipase activity equivalent to 66 acid degrees (culture *b*, 48 hours, table 6). In some instances the differences in morphology were even more pronounced.

#### DISCUSSION

The data show that lipase production by *M. lipolytica* can be influenced markedly by variations in the conditions under which the culture is grown. The absolute values of parallel trials do not agree closely in some instances for reasons as yet unknown, but the data indicate similar trends in quantitative relationships in repetitive trials. Although the data must be considered preliminary because of the many possibilities that were not investigated, the indications are that a complex organic growth medium well buffered at a reaction of about pH 4.5 is most suitable for the production of lipase. The nature of the buffering salts is important. The pronounced effects of variations in the composition of the medium indicate the desirability of further studies of this aspect of the problem.

Temperature has a pronounced effect upon the production of the lipase, apparently principally because of its influence upon cell proliferation. At 36 C the organism fails to grow, and lipase production either is minimal or cannot be demonstrated. At 21 C growth is slow, but both cell population and lipase concentration increase at somewhat comparable rates during the early part of the growth period. In the temperature studies, the total amount of lipase present seems to be partially a function of the time during which the population has been at a high level, e.g., the factor of secretion per cell per unit period of time apparently is important in determining the total enzyme concentration under the circumstances of this portion of the study.

The degree of aeration of the culture determines to a considerable degree the amount of lipase produced, as bubbling sterile air through the culture or shaking the culture repeatedly reduces lipase production, although populations of cells are increased. On the other hand, the addition of reducing agents, such as cysteine hydrochloride, thioglycolic acid, or ascorbic acid, to the culture medium caused no marked increases in lipase production, although the cell populations usually were increased over those of the controls. The addition of these reducing agents quite definitely brought about no increase in enzyme production per cell per unit of time. These data would seem to indicate that somewhat aerobic conditions are needed for lipase production by this organism.

The presence of sugar in amounts exceeding 0.05 per cent proved detrimental to lipase production, a finding which is in agreement with the results of Thibodeau and Macy (1942) in their studies on lipase production by *P. roqueforti*. In the present studies the medium was buffered sufficiently to minimize shifts in pH. The effect of the sugar probably was not that of permitting the building up of detrimental concentrations of specific organic anions, because continued incubation in the medium containing the higher concentrations of sugar resulted in comparatively more favorable lipase production, possibly this was because of the reduction of sugar concentration as the result of use by the organisms, but data upon sugar utilization were not obtained.

Although conditions favorable for optimum growth of the organism failed to produce an accumulation of lipase in the medium, slight modifications of environments resulting in a somewhat retarded growth rate usually were accompanied by an increased amount of lipase in the medium. Whether lipase is produced in all instances, but accumulates only under certain conditions of cultivation of the organism, would require further investigation. The association of higher lipase production with a change in morphology to predominantly longer and more slender cells, as the result of any one of several apparently unrelated modifications of cultural conditions, may indicate a possible common basis for the increased enzyme production. No hint as to what this basis might be was obtained.

By utilizing the available information on factors that influence lipase production by *M. lipolytica*, liquid preparations containing a relatively high concentration of lipase were obtained.

#### SUMMARY AND CONCLUSIONS

Of the five growth media tried, nutrient broth containing 0.05 per cent glucose was most suitable for lipase production by *Mycotorula lipolytica*.

Lipase production by *M. lipolytica* was increased further by the addition of 0.225 M disodium phosphate plus citric acid buffer to the nutrient broth with a reaction of pH 4.5 to 5.5. An equal addition of disodium phosphate plus monopotassium phosphate gave similar high results. A buffer of sodium acetate plus acetic acid was unsatisfactory.

Growth at 30 C was optimum for the production of lipase by *M. lipolytica*.



The accumulation of lipase in the medium was favored by growing the culture without shaking or aeration, but added reducing substances had little or no effect

In general, short oval cells and low lipase activity were associated with conditions favorable for rapid growth, whereas long slender cells and high lipase activity were associated with conditions slightly less favorable for growth

By the utilization of proper cultural conditions, preparations of high lipase activity were obtained from *M lipolytica*

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# PRELIMINARY CHARACTERIZATION OF THE LIPASE OF MYCOTORULA LIPOLYTICA<sup>1</sup>

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In the preceding paper the factors influencing the production of lipase by *Mycotorula lipolytica* were discussed (Peters and Nelson, 1948). The considerable increase in lipase production under proper conditions of growth of the culture warranted a study of the characteristics of the enzyme. This lipase may be of particular interest because of the possibility of its useful application in the cheese-making industry, especially in the manufacture of blue or Roquefort type cheese from pasteurized milk.

Although considerable information is available dealing with the characterization of animal and vegetable lipases, little work seems to have been done on microbial lipases. Avery and Cullen (1920) found that pneumococcus lipase hydrolyzed tributyrin most actively at pH 7.8, whereas Stevens and West (1922) reported that hemolytic streptococci showed greatest lipase activity at pH 7.9, with ethyl butyrate as substrate. The enzyme produced by streptococci was inactivated by exposures to temperatures above 55°C for 5 minutes. Gorbach and Guentner (1932) found the lipase of beer yeast to be most active at pH 6.6 to 6.8. This lipase showed optimum activity at 30°C, with higher temperatures proving detrimental to the enzyme. The same workers also observed that the rate of lipase activity decreased with time and that the amount of olive oil hydrolyzed was not directly proportional to the amount of beer-yeast lipase present, although there was a point of optimum activity per unit of lipase. Thibodeau and Macy (1942) reported that lipase from *Penicillium roqueforti* showed optimum activity at pH 5.3 to 7.5.

The concentration and purification of lipases from various sources has been accomplished by several methods, of which precipitation of the enzyme with certain salts seemed most satisfactory. Gyotoku and Terashima (1930) were able to precipitate lipase of blood, pancreas, and stomach by the use of a 55 to 60 per cent concentration of ammonium sulfate, whereas Gluck and King (1933) used, with varying success, magnesium sulfate, half and fully saturated ammonium sulfate, and 10 per cent sodium chloride as precipitating agents. Ostwald and Mischke (1940) were able to concentrate pancreatic lipase from solutions at pH 4.5 by continuous bubbling of nitrogen gas through the solution and the collection of the foam.

## METHODS

The medium for lipase production, the growth conditions, the determination of pH, and the quantitative determination of lipase activity were the same as reported in an earlier paper (Peters and Nelson, 1948).

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Cell-free preparations were obtained by first centrifuging 3 day-old nutrient broth cultures of *M lipolytica* at 3,500 rpm for 15 minutes and then filtering the supernatant through an ultra-fine sintered glass (pyrex) filter with the aid of suction

The lyophilized material was prepared by placing a 200-ml quantity of a 3-day-old broth culture of *M lipolytica* in a 500-ml round-bottom flask with ground-glass stopper. The culture was frozen while the flask was being rotated in an acetone dry-ice bath. The flask containing the frozen culture was evacuated by the use of a vacuum pump, resulting in sublimation of the ice in the frozen culture. The water vapors were extracted by continuous suction and condensed and frozen in another flask, which was held in an acetone dry-ice bath.

In the concentration of lipase by foaming, 3-day-old broth cultures were centrifuged and the supernatant was placed in 100- or 200-ml quantities into 225- or 500-ml gas-washing bottles, respectively. Foaming was produced by bubbling compressed carbon dioxide gas through the supernatant at from 50 to 75 mm mercury pressure. The foam was collected in graduated cylinders of a size convenient to permit the collection of the desired volume of foam. In the experiments on repeated foaming, the third and fourth fractions were diluted by adding distilled water and acidified with hydrochloric acid to pH 4.0, to obtain the desired volume of 200 ml for further fractionation.

The precipitation of lipase with ammonium sulfate and ethyl alcohol was performed on 3-day-old broth cultures, which were centrifuged and the supernatant used in 400-ml quantities. To 400 ml of supernatant in a 1-liter separatory funnel was added ammonium sulfate to the saturation point (about 200 g). Next, 130 ml of 95 per cent ethyl alcohol were added and the mixture was well shaken. The flocculent precipitate gathered at the water-alcohol interface. After the aqueous layer was drawn off, the precipitate and the alcohol layer were decanted onto a previously weighed hardened filter paper, supported in a Buchner funnel. Suction was applied to the flask and the alcohol filtered off. A 10-ml quantity of diethyl ether was added to remove the residual alcohol. The filter paper was freed from the last traces of ether by placing it at 37°C for 2 hours. The dry precipitate on the filter paper was weighed and its lipase activity measured.

## RESULTS

The data in table 1 show that the pH of the emulsion upon which the enzyme acts has a definite effect upon the activity of the lipase. Although hydrolysis of fat took place over the pH range from 4.0 to 8.0, greatest lipase activity took place at pH 6.2 to 6.5. The decrease in lipase activity was more abrupt on the alkaline side of the optimum than on the acid side.

In five trials in which pasteurized homogenized milk was substituted for butterfat-agar emulsion as substrate (and for which data are not shown), acid degree values ranging between 47 and 87 were obtained, causing a lowering of the reaction of the milk by as much as 0.9 of a pH unit in some cases. These results demonstrate the activity in milk of lipase from *M lipolytica*.

The influence of various incubation temperatures upon the activity of the lipase from *M. lipolytica* is shown in tables 2 and 3. Optimum activity was demonstrated at 28 and 33 C. Inactivation at 43 C is apparent after 30 hours of incubation time, but at 37 C a 54-hour period was required to produce a

TABLE 1  
*The influence of pH upon M. lipolytica lipase activity*

pH OF SUBSTRATE	LIPASE ACTIVITY* PER ML OF ENZYME PREPARATION	
	Trial 1	Trial 2
4.0	16	—
4.5	22	—
5.0	24	—
5.5	26	—
6.0	32	36.5
6.2	40	40
6.4	—	34.5
6.5	55	—
6.6	—	25.5
6.7	37	—
7.0	31	22.5
7.5	15	—
8.0	12	—

\* Expressed in acid degrees

TABLE 2  
*The influence of temperature and time upon M. lipolytica lipase activity*  
(Average of two trials)

REACTION TIME IN HOURS	LIPASE ACTIVITY* AFTER VARIOUS INCUBATION PERIODS AT TEMPERATURES OF			
	28 C	33 C	37 C	43 C
6	11.0	10.5	9.0	12.0
18	20.0	19.5	14.0	15.0
30	22.5	27.5	19.5	16.5
42	35.5	32.5	28.5	18.0
54	43.0	41.0	31.0	18.0
66	45.0	43.0	30.0	17.0
78	49.0	48.5	31.5	20.0
102	62.5	63.0	34.0	20.5
126	70.0	72.5	36.0	23.0
150	74.0	73.5	33.0	19.5

\* Expressed as acid degrees per milliliter of enzyme preparation

distinct inactivating effect. Other trials showed that temperatures above 43 C resulted in earlier inactivation of the enzyme. A temperature of 52 C was sufficient to inactivate the lipase within 8 hours. The initial rate of lipase activity at 10 C was slightly lower than at 21 to 33 C (table 3). However,

results obtained after an incubation period of 810 hours at 10 C showed acid degree values comparable to those of samples held at 21 C. The temperature of 10 C was chosen because blue cheese ripening takes place at approximately this temperature.

The data presented in table 4 show that, when various dilutions of a concentrated enzyme preparation (broth culture concentrated to one-fourth original

TABLE 3

*The influence of lower temperatures and prolonged holding time upon M lipolytica lipase activity*

TIME IN HOURS	LIPASE ACTIVITY* AFTER VARIOUS INCUBATION PERIODS AT TEMPERATURES OF			
	10 C	21 C	23 C	33 C
18	8	12	11	16
66	—	22	23	23
114	13†	27	26	25
162	20	33	29	28
306	27	40	—	—
450	28	29	—	—
810	40	46	—	—

\* Expressed as acid degrees per milliliter of enzyme preparation

† At 90 hours

TABLE 4

*The influence of varying amounts of M lipolytica lipase preparation (concentrated by lyophilizing) upon lipase activity (Average of two trials)*

AMOUNT OF CONCENTRATE USED (ML)	LIPASE ACTIVITY* AS	
	Determined per increment used	Calculated per ml of concentrate used
0.01	5	500
0.03	7	233
0.05	9	180
0.1	15	150
0.2	20	97.5
0.4	32.5	81
0.8	42	52

\* Expressed as acid degrees per milliliter of enzyme preparation

volume by lyophilizing) were used in the quantitative determination of lipase activity and the results calculated back to a basis of total activity per milliliter of original preparation, the calculated values for lipase activity were considerably higher when the smaller quantities of preparation were used.

Storage in well-filled screw-cap test tubes of cell-free filtrates containing lipase showed that a temperature of 3 to 5 C was more satisfactory for the preservation of the enzyme activity than was a temperature 23 to 25 C (table

5) The results of two trials were closely comparable. Other studies showed that the addition of cysteine did not prove advantageous for the preservation of lipase activity during storage.

Concentration of *M. lipolytica* cultures by lyophilizing, either partially or to complete dryness, resulted in considerable concentration of the lipase enzyme, as shown in table 6. However, lyophilized portions equivalent to 1 ml of the original culture yielded acid degree values about one-half of the values of the original culture in two cases, and only one-third of the original value in one case. Continued lyophilizing up to the change from liquid to solid state of the culture resulted in a sticky, gluey mass which was difficult to handle but which

TABLE 5

*The influence of storage temperature, time, and the presence of cysteine upon M. lipolytica lipase activity*

STORAGE CONDITION	LIPASE ACTIVITY* AFTER STORAGE TIME IN DAYS					
	Trial 1			Trial 2		
	0	4	106	0	4	96
3- 5 C	25	22.5	22	32	26	28
23-25 C	25	17.5	13	32	26	16
23-25 C and cysteine	25	21.0	14	32	18	16

\* Expressed as acid degrees per milliliter of enzyme preparation

TABLE 6

*The effect of lyophilizing treatment of M. lipolytica cultures upon its lipase activity*

ITEM STUDIED	TRIAL NUMBER		
	1*	2*	3
Acid degrees per ml of original culture†	36	70	54
Original weight of culture†	200 g	183 g	190 g
Weight of culture after lyophilizing	50 g	31 g	12 g
Acid degrees of 1 ml equiv. of orig. culture	19	24	26
Acid degree ratio of orig. to lyoph. culture	2.1	3.1	2.1

\* Incompletely dried

† Calculated as 1 ml = 1 g

dissolved readily in distilled water or in phosphate buffer solutions, and was possessed of high enzyme activity per unit mass.

Preliminary trials on the concentration of lipase by the foaming procedure at 0.5-unit intervals from pH 4.0 to pH 7.0 gave the highest concentration of lipase in the foam portion and the lowest concentration in the residual portion at pH 4.0. Accordingly, this pH level was used in further experiments on repeated foaming. The results of two trials are shown in table 7. Although the first fractionation did not result in an increase in lipase concentration, as judged by the acid degree values obtained, further fractionations resulted in increases in lipase concentration. Either the removal of the lipase by 50 per cent fraction-

ation was practically complete, or the lipase in the residue was destroyed, since the residues showed little or no lipase activity. The increase in acid degree values in the two trials was approximately 250 per cent in each case. The lipase remaining in the final foam fraction represented about 8 per cent of the initial lipase activity.

In preliminary trials in which magnesium sulfate, ammonium sulfate, and sodium chloride were used as precipitating agents, precipitation of a lipase-active

TABLE 7

*The influence of repeated foaming upon the concentration of lipase produced by *M. lipolytica**

NUMBERS OF FRACTIONATION	VOLUME OF FRACTION (ML)	LIPASE ACTIVITY* PER ML OF			
		Liquid foam		Residue	
		Trial 1	Trial 2	Trial 1	Trial 2
Original	800	27	24	27	21
1	400	20	24	3	4
2	200	26	34	1	3
3	100	40	40	2	0
4	50	50	50	0	4
5	25	67	60	0	1

\* Expressed as acid degrees per milliliter of enzyme preparation

TABLE 8

*The influence of temperature during the salting out with ammonium sulfate and ethyl alcohol upon the lipase activity of the precipitate*

ITEMS STUDIED	TRIAL 1		TRIAL 2	
	6 C	24 C	6 C	24 C
Acid degrees per ml of original supernatant	25	25	23	23
Total weight of precipitate (g)	0.2	0.2	0.16	0.18
Acid degrees per 0.03 g of precipitate	21	6	44	6
Calculated acid degrees per total precipitate	140	40	300	36
Calculated per cent recovery of original total lipase activity	1.4	0.4	3.0	0.36

substance occurred only in saturated solutions of ammonium sulfate. The data in table 8 show the results of two trials in which the lipase-active substance was precipitated by means of ammonium sulfate and ethyl alcohol, as described under "Methods," using temperatures of 6 and 24 C. Although an active preparation was obtained at each temperature, the results are much in favor of the lower temperature. The lipase activities of the precipitate obtained at 6 C were 250 and 730 per cent greater than the activities of the corresponding precipitates prepared at 24 C. In either case the enzyme recovered represented 3 per cent or less of the original lipase activity.



## DISCUSSION

The lipolytic enzyme system of *M. lipolytica* has its optimum activity at pH 6.2 to 6.5, a range that relates the enzyme to the lipases of the yeasts and molds that have been studied in this respect, rather than to the bacterial lipases that are most active at somewhat alkaline reactions. The lipase of *M. lipolytica* is active over the entire range in which blue cheese ripening takes place, namely, pH 4.7 to 6.5 (Coulter, Combs, and George, 1938), and thus might be expected to be able to bring about considerable hydrolysis of butterfat during the ripening process of cheese of this type.

Although the optimum activity of the enzyme upon butterfat is at 28 to 33 C, the considerable activity at 10 C demonstrates the probability that the enzyme would be active in cheese curd at that temperature. The use of temperatures of approximately 32 C, as commonly maintained during the cheese-making process until hooping of the curd, would favor early enzyme activity.

Inactivation at temperatures of 42 C and above should result in no difficulty in cheese making as long as proper temperature levels are maintained during the cheese-making operation following the addition of the enzyme to the pasteurized milk. In a similar manner, the storage of cell-free enzyme preparations at low temperatures, such as 3 to 5 C, should aid in maintaining the strength of the lipase in solution, although a slight drop in activity may be expected on prolonged holding.

With respect to the concentration and purification of the enzyme system, all three methods employed resulted in large losses of total lipase activity. Lyophilizing was least detrimental to the enzyme, whereas fractionation by either foaming or salting-out at room temperature resulted in the greatest losses of lipase. The use of lower temperatures during foaming might very possibly decrease the extent of inactivation during the process, as was observed in the trials on salting-out of lipase with ammonium sulfate and ethyl alcohol. Further work would be necessary in order to obtain a more complete picture of this situation.

The observed characteristics of the enzyme indicate that it may be of considerable value in the manufacture of blue or Roquefort-type cheese from pasteurized milk as a replacement for the normal milk lipase destroyed in pasteurization.

## SUMMARY AND CONCLUSIONS

Lipase activity on butterfat over a range of reaction from pH 4.0 to pH 8.0 was demonstrated for lipase obtained from *Mycotorula lipolytica*, with pH 6.2 to 6.5 being optimum.

Lipase activity on butterfat was demonstrated at temperatures from 10 to 52 C, temperatures of 37 C and above inactivated the enzyme, the reaction being more rapid at the higher temperatures studied.

A storage temperature of 5 C was much superior to 25 C for preserving the enzyme in cell-free preparations, activity being maintained at a high level for at least 3 months when storage was at the lower temperature in closed containers.

Lyophilized enzyme preparations retained about half their original total lipase activity

Considerable concentration of lipase by foaming was demonstrated, large losses of enzyme activity occurred during the process

The salting-out of lipase with saturated ammonium sulfate plus 30 per cent ethyl alcohol resulted in a white precipitate high in lipase activity

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## NUTRITION OF STREPTOCOCCUS BOVIS

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A study was undertaken to determine the nutritive requirements of certain strains of *Streptococcus bovis* that have been isolated from both bovine and human sources. These strains have been found to differ somewhat with respect to their action on blood agar from the so-called "typical" *Streptococcus bovis* found commonly in the alimentary tract of cows. The bovine strains used in this study were isolated from the throats of young calves, but similar types also occur frequently in the intestines of cows. The human strains were isolated from cases of subacute bacterial endocarditis.

It was found that this organism has the simplest nutritive requirements of any of the streptococci thus far studied.

### CULTURES AND TECHNIQUE

The cultures employed throughout the study consisted of 8 human strains isolated from subacute bacterial endocarditis and 7 bovine strains isolated from the throats of young calves. The physiological characteristics of these organisms are given in table 1. It should be noted that these organisms are of the "indifferent" type on blood agar, whereas most strains of the "typical" *Streptococcus bovis* produce an alpha or greening reaction.

The technique used throughout this study was essentially the same as that previously reported (Smiley, Niven, and Sherman, 1943). A 24-hour meat infusion broth culture was used for inoculation, and transfers into the experimental media were made using a fine wire needle. Whenever growth occurred, one or more subcultures were made into the same medium at 24-hour intervals.

### GROWTH FACTOR REQUIREMENTS

The basal medium used in determining the growth factor requirements was composed of 0.5 per cent acid-hydrolyzed, vitamin-free casein, 1.0 per cent glucose, 0.6 per cent  $K_2HPO_4$ , 10 mg per cent sodium thioglycolate, 10 mg per cent L-tryptophan, 10 mg per cent L-cystine, and salts (table 3). The medium was adjusted to pH 7.2, tubed in 5-ml quantities, and autoclaved at 15 pounds pressure for 15 minutes. The cultures were incubated at 37 C and the growth response was measured with a photoelectric densitometer at approximately 24- and 48-hour intervals.

No observable growth occurred in the basal medium alone, but the addition of the known vitamins of the B complex afforded luxuriant growth. Upon the elimination of each of the vitamins individually, it was found that biotin was

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required for the initiation of growth of all strains tested. Thiamine was found to stimulate growth greatly, although all strains could be successfully subcultured in a medium that did not contain this vitamin. That the medium was not being contaminated with thiamine was demonstrated by the fact that it would

TABLE 1  
*Physiological characteristics of Streptococcus bovis strains*

CHARACTERISTICS	HUMAN (8 STRAINS)	BOVINE (7 STRAINS)
Blood agar	Indifferent	Indifferent
Growth		
10 C	—	—
45 C	+	+
40% bile	+	+
6.5% NaCl	—	—
Survives 60 C, 30 min	+	+
Curdles milk	+	+
Strong reducing action	—	—
Final pH, glucose broth	4.0-4.2	1.0-4.2
Hydrolysis		
Starch	+	+
Arginine	—	—
Sodium hippurate	—	—
Esculin	+	+
Mucoid colonies, sucrose agar	+	+
Fermentations		
Arabinose	—	6+, 1—
Xylose	—	—
Glucose	+	+
Maltose	+	+
Lactose	+	+
Sucrose	+	+
Trehalose	+	+
Raffinose	+	+
Inulin	6+, 2—	+
Glycerol	—	—
Mannitol	+	+
Sorbitol	—	—
Salicin	+	+

\* Colonies were occasionally mucoid, but not markedly so. Large quantities of polysaccharide were synthesized in 5 per cent sucrose broth, as evidenced by a marked increase in the viscosity of the medium.

not support the growth of *Streptococcus salivarius* without the addition of thiamine to the medium.

Nicotinic acid was found to have a slight stimulatory action on the growth of these organisms, whereas some variability was demonstrated in the case of pantothenic acid. Two strains of bovine origin required this vitamin for subculture, although all other strains tested were only slightly stimulated by it.

The response to riboflavin was also variable. Two strains of bovine origin were found to require this vitamin, although it produced no observable effect upon the growth of the other strains tested. The absence of pyridoxine from the medium had no perceptible effect upon the growth of any of the cultures tested. The elimination of the purine and pyrimidine bases from the medium produced little effect upon the growth of this organism.

The growth response resulting from the elimination of vitamins individually from the medium, showing the results obtained with a typical strain of bovine and one of human origin, is given in table 2.

It should be mentioned that those strains that showed an absolute requirement for only one vitamin, biotin, when the vitamins were eliminated from the casein hydrolyzate medium individually, could not be serially transferred in a medium containing only biotin and thiamine. Good growth was usually obtained on the first transfer. The second serial transfer, however, produced

TABLE 2

*The effect of omitting vitamins upon the growth of Streptococcus bovis,  
[P10 (human) and 38 (bovine)]*

VITAMIN(S) OMITTED	GROWTH 24 HOURS*	
	P10	38
None	210	190
Thiamine	38	68
Biotin	2	0
Nicotinic acid	185	150
Pantothenic acid	140	125
Riboflavin	240	190
Pyridoxine	250	185

\* Uninoculated medium reads zero. Readings made on second serial transfer.

little or no growth. For maximum growth that could be maintained on serial transfer, biotin, thiamine, pantothenic acid, and nicotinic acid were required.

These strains of *Streptococcus bovis* appear to have far simpler vitamin requirements than any of the other nonhemolytic streptococci thus far tested; they have an absolute requirement for only one vitamin, biotin. In contrast to this, it has been shown (Smiley, Niven, and Sherman, 1943) that 5 vitamins are indispensable for the growth of *Streptococcus salivarius*, and that most strains of *Streptococcus lactis* have an absolute requirement for at least 4 vitamins (Niven, 1944). Furthermore, it has been demonstrated (Niven and Sherman, 1944) that the enterococci are even more exacting in their vitamin requirements than either of these organisms.

#### AMINO ACID REQUIREMENTS

After determination of the growth factor requirements, the hydrolyzed casein was substituted by a mixture of 20 amino acids. By the elimination of each of

these amino acids individually from the medium it was found that, with the exception of tryptophan, the omission of any single amino acid produced no detectable effect upon the growth of these organisms. When tryptophan was omitted from the amino acid medium, or from the medium containing acid-hydrolyzed casein, these microorganisms often failed to grow. However, for reasons unknown at the time, delayed growth would occur occasionally in media containing no tryptophan. However, subsequent experiments revealed that, in media containing certain combinations of only a few amino acids, tryptophan was no longer essential for growth. This phenomenon is discussed in another publication (Washburn and Niven, 1948).

By the systematic elimination of various combinations of amino acids it was found that the majority of the strains could be successfully subcultured in a

TABLE 3  
*A chemically defined medium for Streptococcus bovis*

	mg/10 ml
Glucose	100
K <sub>2</sub> HPO <sub>4</sub>	60
Sodium thioglycolate	5
D-Arginine	5
D-Glutamic acid	1
	μg/10 ml
Nicotinic acid	10
Pantothenic acid	10
Thiamine	1
Biotin	0.01
Salts	•

\* Salts composed of 20 mg NaCl, 0.8 mg MgSO<sub>4</sub> · 7H<sub>2</sub>O, 40 micrograms FeSO<sub>4</sub> · 7H<sub>2</sub>O, and 12 micrograms MnCl<sub>2</sub> per 10 ml medium

medium containing arginine as the only source of amino nitrogen. The addition of glutamic acid to the medium resulted in the growth of all strains except one culture of bovine origin.

This group of organisms, therefore, is unusual among the streptococci thus far studied in its ability to synthesize amino acids, since it has an absolute requirement for no single amino acid. *Streptococcus salivarius* (Smiley, Niven, and Sherman, 1943) appears to have an absolute requirement for only one amino acid, glutamic acid. However, 7 amino acids are necessary for satisfactory transferable growth of that organism.

A simplified medium for the growth of *Streptococcus bovis* is shown in table 3. It contains 4 vitamins and 2 amino acids. Twelve of the 15 cultures studied grew satisfactorily in this medium upon serial transfer. By the addition of riboflavin all but one culture (of bovine origin) would grow satisfactorily for an indefinite number of transfers.

## SULFUR REQUIREMENTS

It should be noted in table 3 that sodium thioglycolate is the only source of organic sulfur in the simplified medium, other than that supplied by the vitamins. Since the sulfur requirements of this organism appear to be unusually simple, an attempt was made to determine whether inorganic sulfur could be substituted for the organic sulfur in the medium. A number of sulfur-containing inorganic compounds were tried, including sodium sulfide, sodium sulfite, and ammonium sulfate.

It was found (table 4) that inorganic sulfur in the form of sodium sulfide gave maximum growth of the organism, but sodium sulfite and ammonium sulfate were ineffective. That certain streptococci can utilize sodium sulfide as their source of sulfur was previously demonstrated by Woolley (1941). Using a hemolytic group D streptococcus, he found that, although cystine was more effective, sodium sulfide could meet the sulfur requirements of that organism.

TABLE 4  
*Streptococcus bovis* sulfur requirements

SUBSTANCE ADDED	MG/10 ML	GROWTH	
		18 hr	44 hr
Sodium thioglycolate	1	45	80
Sodium thioglycolate	5	125	155
Na <sub>2</sub> S	1	68	180
Na S	5	49	175
Na <sub>2</sub> S	10	53	375
Na <sub>2</sub> SO <sub>3</sub>	1	0	0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1	0	0
Control		0	0

## SUMMARY

The nutritive requirements of certain strains of *Streptococcus bovis* have been determined. These organisms were found to be unique among the streptococci in the simplicity of these requirements.

A majority of the strains tested have an absolute requirement for only one vitamin, biotin, whereas thiamine stimulates growth appreciably. Nicotinic acid and pantothenic acid slightly stimulate the growth of most strains. However, to obtain maximum growth and serial transfer in a synthetic medium, it was necessary to add all four of these vitamins.

No single amino acid is indispensable to the growth of *Streptococcus bovis*. Each strain tested, except one, could be subcultured in a medium containing arginine and glutamic acid as the only amino acids present.

An inorganic source of sulfur in the form of sodium sulfide fulfills the sulfur requirements of this organism.

No difference was found in the nutritive requirements of the bovine strains and the strains obtained from human cases of subacute bacterial endocarditis, though slight strain differences were detected in the cultures of each group

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# ANTIBIOTIC SUBSTANCES SEPARATED FROM SUMAC<sup>1</sup>

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It has become evident (Osborn, 1943, Lucas and Lewis, 1944) that higher plants are potential sources of antibiotic agents. Several investigators (Little and Grubaugh, 1946, Atkinson and Rainsford, 1946, Sanders, Weatherwax, and McClung, 1945) have reported antibiotic effects of juices and of crude extracts of plants on various microorganisms. The active materials can be separated in some degree of purity (Cavallito, Buck, and Suter, 1945, Cavallito, Bailey, and Kirchner, 1945, Heatley, 1944), and there is evidence that the agents so obtained can be introduced into the animal body and will control experimental infections (Carlson, Bissell, and Mueller, 1946).

In screening several hundred plants (Carlson, Douglas, and Robertson, 1948) the authors observed that a species of sumac contained an antibiotic agent extractable in aqueous solutions. In later work this or similar agents were obtained from sumac by extraction with ethyl ether. It is the purpose of this paper to report the antibiotic activity of partially purified extracts from a species of sumac, *Rhus hirta*.

## METHODS

*Chemical procedures* Stems and leaves collected in the fresh state were finely chopped and sufficient ethyl ether was added to cover the material. Extraction was carried out for 24 to 48 hours at room temperature. The ether extraction (911B1) was separated from the plant residue by decanting. The plant residue was then allowed to stand at room temperature for 24 to 48 hours with an equal volume of distilled water (see flow chart, figure 1). The supernatant water extract (911B90) was decanted from the plant residue and was evaporated to dryness, yielding a brown, brittle material (911B96). This material was partially soluble in distilled water as a red solution (911B97) with the persistence of some red-gray precipitate (911B98). Ten volumes of ethyl alcohol (95 per cent) were added to the red solution. A clear yellow supernatant and a gray precipitate (911B100) were obtained. The supernatant was evaporated to remove the alcohol. A dark brown solution remained (911B102).

The ether was evaporated from the original extraction (911B3) leaving a green gum (911B15). The gum was placed in a Soxhlet extraction thimble and extracted with chloroform until colorless (approximately 5 hours). The chloroform was decanted and evaporated again leaving a thick green gum (911B50). The residue (911B47A), after chloroform extraction, was divided

<sup>1</sup> Supported in part by a U S Naval grant, Office of Naval Research

into two portions and re-extracted with ethyl ether and water (911B94). The residue (911B55) remaining in the extraction thimble was re-extracted with distilled water, yielding a clear brown solution (911B71). The ether extract was evaporated to dryness, yielding a greenish-brown gum (911B47). This gum

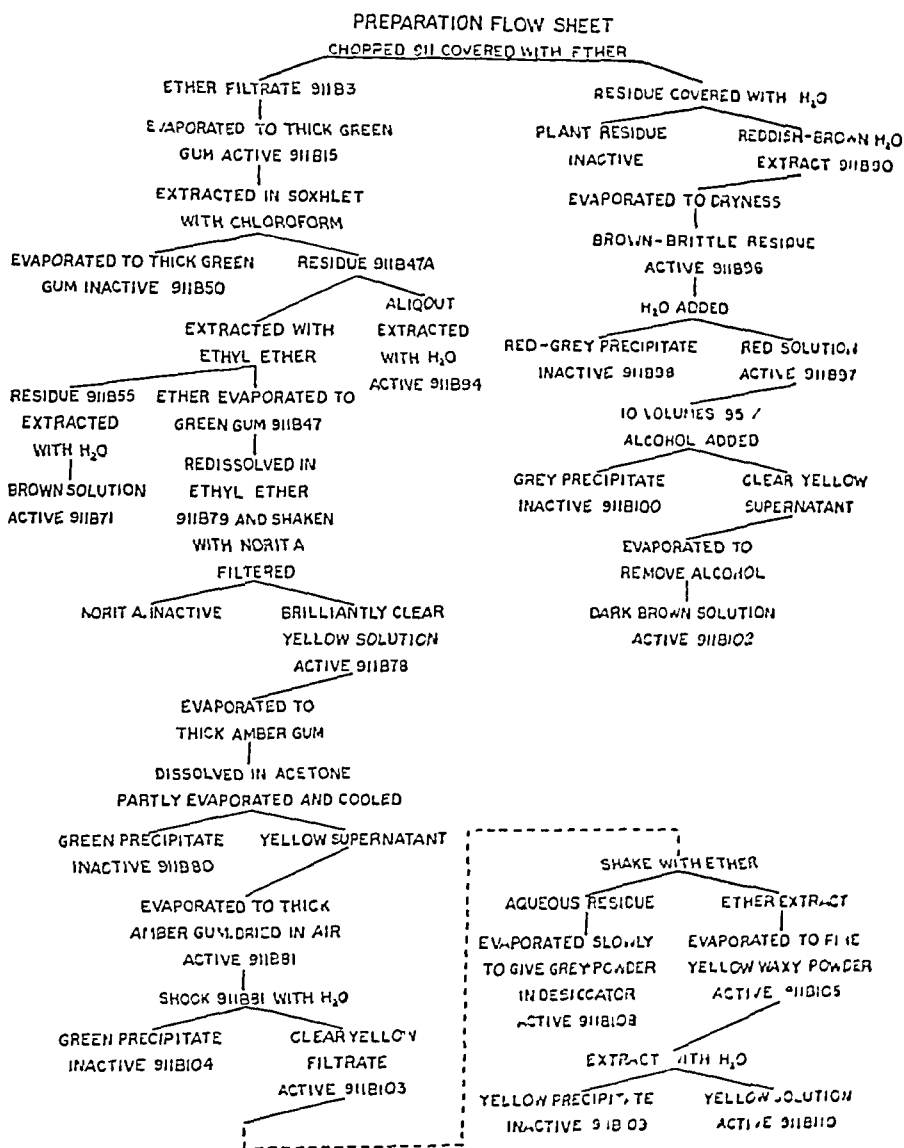


FIG 1 FLOW CHART

was redissolved in ethyl ether (911B79) and shaken with 2 parts norit A. The resultant solution (911B78) was a clear, brilliant yellow. This was evaporated until a thick amber gum was obtained. The gum was dissolved in acetone and the solution evaporated to one-tenth its original volume. The green pre-

precipitate (911B80) that formed was removed by filtration. The filtrate was evaporated to a thick green syrup (911B81), which was dried to a solid mass and pulverized. This powder (911B81) was shaken with distilled water and the suspension filtered. A green precipitate (911B104) was removed in the paper, and the filtrate (911B103) was a clear, yellow solution. The filtrate (911B103) was shaken for 15 minutes with ethyl ether. After being shaken, the ether was decanted and evaporated to dryness. A fine yellow, waxy powder (911B105) was obtained. This powder was partially soluble in distilled water, yielding a yellow solution (911B110), but there remained a yellow insoluble precipitate (911B109) that was removed by filtration. From the aqueous residue of the ether extraction of 911B103 there developed on slow evaporation an amber solution containing a whitish granular precipitate. This precipitate (911B108) became a gray powder on desiccation.

**Assay** Nutrient and enriched agar (Difco), 20 ml, favorable for the growth of the organisms on test, was seeded with 0.5 ml of an 18- to 24-hour broth culture of the organisms. Fungi and mold cultures were thoroughly agitated to break up mycelium formations before addition to the agar. After mixing, the agar was poured into sterile petri plates. The surface of the seeded agar plates was allowed to dry in air for 30 to 60 minutes. Several species of bacteria were grown on blood agar. One-tenth ml of a broth culture was placed on the surface and spread evenly with a curved sterile glass rod. These plates were allowed to dry for 30 to 60 minutes. Porcelain cylinders were placed on the surface of the seeded plates and were filled with the aqueous solutions to be tested for antibiotic activity. In a second method small blocks of agar were removed from the plate surface by vacuum, and the wells so formed were filled with the aqueous solutions.

Dry powders and gums were placed directly on the surface of the seeded agar. Ether extracts were tested as previously described (Carlson, Douglas, and Robertson, 1948). The areas of inhibition were measured in mm whenever possible. In those areas where the diffusion of the active agent was uneven, inhibition was noted as negative (N), slight—8 to 11 mm (S), fair—12 to 14 mm (F), good—15 to 19 mm (G), or excellent—20+ mm (E). The letter "C" preceding any number or abbreviation denotes complete inhibition and "P" denotes partial inhibition.

**Toxicity** Extracts 911B94, 911B102, and 911B108 were prepared in 10 per cent solutions and passed through a Seitz filter. The sterile extracts were injected in 0.25, 0.5, and 1 ml amounts by the intraperitoneal route. Adult white Swiss mice were used as test animals. Toxicity was observed at intervals of 15 and 45 minutes and at the end of 1 and 24 hours.

#### RESULTS

The antibiotic activity of the extracts and fractions prepared during partial purification is shown in table 1 (also see flow chart). The different extracts appeared to be equally effective against the gram-positive coccus, *Staphylococcus aureus*, and the gram-negative organism, *Escherichia coli*. One extract, 911B15,

a gum obtained from ether evaporation, stimulated the test organisms, as indicated by a more dense growth immediately surrounding the inhibited zones. The effective agents appeared to be soluble equally in ethyl ether and water.

As observed in the preparation flow sheet, two original extracts, water soluble and ether-soluble, were prepared. These extracts were prepared to yield extract 911B102, a dark-brown aqueous solution from the water-soluble fraction, and aqueous extracts 911B108 and 911B110 derived from the original ether fraction. The effective agent obtained in the aqueous fraction after ether extraction appeared to have been bound in some manner as it had not been apparent before.

TABLE 1  
Antibiotic activity of extracts\*

EXTRACT	SOLVENT	INHIBITION MM	
		<i>S. aureus</i>	<i>E. coli</i>
911B90	Water	C15P25	C15P25
911B96	—(a)	C15P22	CPED
911B97	Water	C14P20	C18P24
91B102	Water	C14P20	C18P24
911B3	Ether	CFD	CPFD
911B15	—(b)	CGDS	CEDS
911B94	Water	C16P24	C16P30
911B71	Water	C20P30	C20P25
911B78	Ether	C15P25	C20P30
911B81	—(a)	C17P28	C18P28
911B103	Water	C13	C17
911B108	Water	C13P20	C14P18
911B105	—(a)	CGD	CGD
911B110	Water	C17	C19

Legend (a)—residue or powder, (b)—gum, C—complete, P—partial, ED—fair diffusion, 12–14 mm, GD—good diffusion, 15–19 mm, ED—excellent diffusion, 20 plus mm, S—stimulation outer area of inhibition.

\* See flow chart (figure 1)

this procedure. Aqueous extraction of the plant alone did not demonstrate the water-soluble agent when tested on the seeded agar plate.

The antibiotic activity of three extracts of *Rhus hirta* against 58 strains and species of bacteria, molds, and fungi is shown in table 2. Extracts 911B102, 911B94, and 911B108 (see flow chart) were used. Extract 911B94 is a step fraction of the partially purified fraction 911B108. The general activity of these three extracts is quite similar in that the effective agents inhibited equally well the growth of the microorganisms tested. Bacteria were observed to be more susceptible to the agent than were the various molds and fungi.

The extracts were observed to exhibit marked bacteriostatic activity on the growth of gram-negative bacteria. Among those tested were several human pathogens, *Shigella paradysenteriae* (Flexner), *Shigella dysenteriae*, *Shigella*

TABLE 2  
Bacterial, mold, and fungus spectrum

ORGANISM	INHIBITION MM		
	911B102	911B94	911B103
<i>Shigella paradysenteriae</i>	C15	C17	CAE
<i>Shigella dysenteriae</i>	C20	C16	CFD
<i>Shigella sonne</i>	C18P22	C15	C16
<i>Eberthella typhosa</i>	C20P26	C17	C15P22
<i>Pseudomonas fluorescens</i>	C18P22	C15P17	CPGD
<i>Pseudomonas aeruginosa</i>	C15P22	C14P18	CPGD
<i>Proteus</i> sp	C20P32	C22P33	C17P27
<i>Proteus vulgaris</i>	C20P28	C18P22	C16P20
<i>Bacillus subtilis</i>	C20P23	C21	C23
<i>Bacillus megatherium</i>	C20P28	C24P30	C17P20
"Zoogloeal" sp	—	C22	C24
<i>Achromobacter lacticum</i>	C16P25	C15P18	C16P20
<i>Serratia marcescens</i>	C20P22	C18P28	PAE
<i>Bacillus circulans</i>	C30	C27	C24
<i>Escherichia coli</i> 0	C15P24	C15×18	CAE
<i>Escherichia coli</i> 1	C16P22	C16P24	C17P29
<i>Escherichia coli</i> S A W	C14P20	C15	CAE
<i>Staphylococcus aureus</i> 0	C20P22	C16P20	C9P18
<i>Staphylococcus aureus</i> 1	C18P24	C16P18	C12P18
<i>Staphylococcus aureus</i> 2	C16P25	C16P20	C10P20
<i>Streptococcus viridans</i>	C17	C11(b)	C15P18
<i>Streptococcus viridans</i>	C13P15	C15(b)	C12P15
<i>Streptococcus pyogenes</i>	N	N	C14
<i>Streptococcus faecalis</i>	—	C20	C14P20
<i>Diplococcus pneumoniae</i>	—	N	N(a)
<i>Clostridium perfringens</i>	N*	N*	N*
<i>Clostridium putrificum</i>	P*	P*	P*
<i>Clostridium histolyticum</i>	N*	N*	N*
<i>Clostridium botulinum</i>	P*	N*	N*
<i>Clostridium sporogenes</i>	N*	N*	P*
<i>Micrococcus tetragenus</i>	C15S24	C14	C15
" <i>Mucosus capsulatus</i> "	C25	C22	C20
<i>Corynebacterium diphtheriae</i>	C18P25	C18P25	CPGD
<i>Mycobacterium phlei</i>	—	C20	C18
<i>Mycobacterium smegmatis</i>	—	C25	C35×40
<i>Neisseria gonorrhoeae</i>	C30	C20	C20
<i>Neisseria intracellularis</i>	C20	C18	C20
<i>Hemophilus influenzae</i>	C20	C16	P24
<i>Agrobacterium</i> sp	—	C18P23	C20P25
<i>Mycoderma lactis</i>	C15	C18P20	CAE
<i>Mycoderma</i> sp	N	C18	CAE
<i>Penicillium</i> (Waksman)	N	N	N
<i>Aspergillus terreus</i>	N	N	N

C—complete, P—partial, S—stimulation, FD—fair diffusion, GD—good diffusion, AE—area exposed, N—negative, a—partial inhibition in broth, b—complete inhibition in broth

\* Broth

TABLE 2—Continued

ORGANISM	EXHIBITION MM		
	911B102	911B94	911B103
<i>Aspergillus niger</i>	N	N	N
<i>Trychophyton</i> sp	N	CFD	CFD
<i>Trychophyton sulphureum</i>	C10	C15P18	C15
<i>Candida albicans</i>	N	C20	CAE
<i>Microsporium trichoderma</i>	P12	C12	CAE
<i>Penicillium cyclopium</i>	C23	C24	C15
<i>Pythium debaryanum</i>	C24	C17	C12P18
<i>Fusarium culmorum</i>	C24	C20	C17
<i>Fusarium</i> sp	N	C17	C20
<i>Rhizoctonia solani</i>	N	N	N
<i>Rhizoctonia oryzae</i>	N	N	N
<i>Pestalotzia funera</i>	—	C19P25	C20
<i>Mucor sylvaticus</i>	—	C17P27	C16

sonne, and *Eberthella typhosa* of the enteric group *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus* sp, *Bacillus circulans*, and 3 strains of *Escherichia coli* were tested, and these nonpathogens were equally susceptible. *Neisseria gonorrhoeae*, *Neisseria intracellularis*, and *Hemophilus influenzae* were inhibited by the three extracts in moderate zones of diffusion.

The gram-positive bacteria tested did not consistently exhibit susceptibility to the effective agents in these extracts. *Bacillus subtilis*, *Bacillus megatherium*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus viridans*, *Streptococcus faecalis*, *Micrococcus tetragenes*, and "*Mucosus capsulatus*" were completely inhibited in varying zones of diffusion. *Streptococcus pyogenes* was only susceptible to 911B108. *Diplococcus pneumoniae* was not inhibited, when grown on blood agar, by extracts 911B94 and 911B108, though an apparent reduction of growth was noticed in broth cultures of this microorganism in the presence of 911B108. Of the five strains of *Clostridium* tested, only one, *C. putrificum*, was susceptible to the three extracts, and it was only partially inhibited, *C. botulinum* was partially inhibited by extract 911B102, and *C. sporogenes* was partially inhibited by extract 911B108. Two strains of nonpathogenic *Mycobacterium*, *M. phlei* and *M. smegmatis*, were very susceptible to the effective agents in extracts 911B94 and 911B108.

Extract 911B102 was ineffective against the growth of three human dermatophytes, *Mycoderma* sp, *Trychophyton* sp, and *Candida albicans*. These organisms were sensitive to extracts 911B94 and 911B108. The extracts appeared to be without significant action against the molds, *Aspergillus terreus*, *Aspergillus niger*, and *Penicillium* (Waksman), but they exhibited marked fungistatic activity toward *Penicillium cyclopium*. *Rhizoctonia solani* and *Rhizoctonia oryzae* were not susceptible to the antibiotic activity of the three extracts. Extract 911B102 was without significant effect upon a plant-wilt pathogen, *Fusarium* sp, though extracts 911B94 and 911B108 were observed to

elicit definite inhibitory activity against this microorganism *Fusarium culmorum* was inhibited equally well by the three extracts

† Human dermatophytes, *Mycoderma lactis*, *Trychophyton sulphereum*, and *Microsporium trichoderma*, and phytopathogens, *Pythium debaryanum*, *Pestalotzia funera*, and *Mucor sylvaticus*, were found to be susceptible to the fungistatic action of the extracts

The extracts were partially inactivated after being subjected to a temperature of 123 C for 10 minutes. Only surface growth was completely inhibited in the diffused area against the gram-positive organism, *Staphylococcus aureus*. No activity against gram-negative organisms was observed with the heat-treated solutions. Extract 911B108 lysed red blood cells, but 911B94 and 911B102 had no apparent effect on the red blood cells.

**Toxicity tests** A 10 per cent solution of extract 911B102 when injected intraperitoneally into mice in 1-, 0.5-, and 0.25-ml amounts did not appear to produce toxic symptoms. Extract 911B94 in 1-ml amounts caused death of the animals in 45 minutes, whereas 0.5- and 0.25-ml amounts caused death in 18 to 20 hours. Extract 911B108 was found to be toxic, causing death in 18 to 20 hours when 1-, 0.5-, and 0.25-ml amounts were injected intraperitoneally.

#### DISCUSSION

The cause of the whitish opacity when extracts of this plant were placed on agar is not known. Tests conducted to determine the presence of alkaloids have proved negative. The second area of discoloration observed in the presence of many of the bacteria tested was deemed to be caused by by-products of the organisms reacting with the diffused agent in a manner similar to an indicator.

In the original screening and later experiments the authors were not able to demonstrate sufficient activity in water or saline extracts to justify further work on this fraction. This did not hold true for extractions with ethyl ether. The aqueous residue after ether extraction showed definite antibiotic activity that was diffusible and measurable on the seeded agar plate. This water-soluble fraction appeared to be released by extraction with ether or possibly was changed chemically to become soluble in water by the treatment.

The two partially purified derivatives, 911B102 and 911B108, appear to be rather closely related agents because of their observed effectiveness against the bacteria tested, although their reaction in the presence of molds and fungi places more emphasis on the probability that they are different substances derived from the same plant.

Sufficient chemical analysis has not been completed to state of what these effective agents are composed. Tests have shown that the gums and water-soluble fractions give no positive tests for alkaloids.

#### SUMMARY

The antibiotic activities of three extracts obtained from *Rhus hirta* are presented. These extracts were tested against 58 different strains and species of

bacteria, molds, and fungi The activity against these microorganisms indicates that this plant contains two separate antibiotic substances

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# ANTIBIOTIC AGENTS SEPARATED FROM THE ROOT OF LACE-LEAVED LEPTOTAENIA<sup>1</sup>

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The presence of antibiotic substances in plants, especially the green parts and flowers, has been demonstrated in a large series of wild and domestic varieties by the authors (Carlson, Douglas, and Robertson, 1948) and by other investigators (Osborn, 1943, Lucas and Lewis, 1944, Huddleson *et al*, 1944). Few plants have been found to contain appreciable amounts of antibacterial and antifungal substances in the roots.

In preliminary screening studies (Carlson, Douglas, and Robertson, 1948), the root of *Leptotaenia dissecta* Nutt (Peck, 1941), belonging to the *Umbelliferae* or parsley family, was found to contain an antibiotic agent soluble in ethyl ether that inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*. It is the purpose of this paper to report additional studies on the effectiveness against various microorganisms of extracts prepared from the root.

## METHODS

The root of *Leptotaenia dissecta* was collected on the western slope of the Cascade Mountains in Oregon and shipped to the laboratory, where it was stored in the cold room until used. Several large specimens of the root were macerated thoroughly by being ground in a food chopper. The macerated root was then placed in a large flask, and flowing steam was circulated through the root mash. The steam distillation was continued for a period of 18 to 24 hours. A brilliant, clear, light-yellow oil came to the surface of the condensate. The oil was removed by decanting and was used for tests without further purification.

Nutrient and enriched agars (Difco), 20 ml, were inoculated with 0.1 ml of an 18- to 24-hour broth culture of the various microorganisms tested (table 2). Broth cultures of molds and fungi were first vigorously shaken to break up the mycelia. The seeded agar was poured into sterile petri dishes, and the surface was air-dried for 30 to 60 minutes.

The method of testing plant extracts previously described (Carlson and Douglas, 1948) was found to lead to a false evaluation of the effectiveness of the oil whether it was placed in a porcelain cylinder or directly on the seeded agar. The latter method had been used by the authors for testing the activity of extracts in volatile solvents. After several preliminary inhibition assays, the following method was found to give consistent results. Small blocks of agar were punched out from the seeded plates with a sterile 8-mm cork borer. The

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bore was attached to the vacuum line with a minimum of negative pressure applied. Excessive negative pressure resulted in an irregular hole in the agar. In the well so formed, several drops (1 to 3) were placed. The inoculated petri plates were incubated at room (29 C) or incubator (38 C) temperature until growth of the microorganisms was sufficiently advanced to allow accurate measurement of the zones of inhibition (24 to 120 hours). Areas of inhibition were noted as complete (C) and partial (P), and diffusion zones as slight (8 to 14 mm), fair (14 to 20 mm), good (20 to 30 mm), and excellent (30+ mm).

A series of dilutions was prepared from one of the extracts (657B30) to permit some degree of qualitative appraisal of the bacteriostatic and bactericidal effect. One-tenth ml of the oil extract was added to 0.9 ml of sterile mineral oil. The mixture was thoroughly agitated, and 0.1 ml was transferred to a second tube containing 0.9 ml of mineral oil. This process was repeated until a final dilution of the original oil fraction was  $10^{-6}$ . To each tube 0.05 ml of a 1:100 dilution of broth culture of *Staphylococcus aureus* or *Escherichia coli* were added. This broth-oil mixture when thoroughly shaken formed an unstable suspension. Shaking was repeated when the mixture separated into layers. One series was set up to include broth cultures shaken in the oil extract of 657B30 undiluted, in dilutions of 1:10 and 1:100, and in mineral oil controls. One standard 4-mm loop of the oil-organism suspension was removed at 5-, 15-, and 30-minute intervals, and at the end of 1 hour and 18 hours, and transferred to veal infusion broth (Difco). These cultures were incubated at 37 C. The presence of growth was observed after 24 and 48 hours. Subcultures from these tubes were frequently made to ascertain whether residual concentrations of oil were carried over. A second series of oil dilutions varying from  $10^{-1}$  to  $10^{-6}$  were inoculated in the same manner as previously described with the same test organisms. Subcultures of the oil-organism suspensions were made at the end of 24 and 48 hours.

To determine toxicity, fraction 657B30 was mixed in sterile mineral oil in concentrations of 1:3 and 1:100. These mixtures in 0.25- and 0.5-ml amounts were injected subcutaneously and intraperitoneally into young adult (4- to 5-week-old) white Swiss mice. All the animals were observed at the end of 10 and 30 minutes and at 1-, 6-, and 24-hour intervals.

#### RESULTS

Quantitative determinations of the amount of active oil extracted from the root were not made nor can it be stated whether different lots of the root would yield the same amounts of oil. When 400 grams (wet weight) of the root *Leptolaena dissecta* were subjected to steam distillation, the first 400 ml of distillate yielded a few drops of a colorless oil that had a sharp odor different from the aroma of the original root. The next 400 ml of distillate brought out 0.7 ml of a yellow-green oil that gave the characteristic "parsnip" odor of the root. Owing to the "spreading" tendency of the oil some was lost during collection. With 18 to 24 hours' steam distillation of large amounts of macerated root a clear yellowish oil was obtainable that was characterized by a definite "parsnip" odor. The oil gradually discolored when exposed to air at room temperature.

The first colorless oil fraction was observed to inhibit completely *Staphylococcus aureus* and *Escherichia coli*, with excellent diffusion of the active agent. The yellowish oil obtained by longer periods of distillation acted in the same manner, but a smaller area of diffusion was noted. No antibacterial vapor effect could be demonstrated with either sample when tested against *Staphylococcus aureus* and *Escherichia coli*.

Table 1 depicts the bactericidal activity of fraction 657B30 alone and diluted in sterile mineral oil. The test organisms in a 1:100 broth suspension were shaken with the oil samples. The undiluted oil fraction was bactericidal for *Escherichia coli* and *Staphylococcus aureus* after 5 minutes' contact with or without constant shaking. No evidence of growth in broth cultures was observed in samples removed after 1 hour's contact with the 1:10 solution of the oil in mineral oil. With cultures of oil in mineral oil the 1:100 dilution required 18

TABLE 1  
Bactericidal effect of fraction 657B30

TIME		DILUTION														MINERAL OIL	
Min	Hr	0		10 <sup>-1</sup>		10 <sup>-2</sup>		10 <sup>-3</sup>		10 <sup>-4</sup>		10 <sup>-5</sup>		10 <sup>-6</sup>		S	C
		S	C	S	C	S	C	S	C	S	C	S	C	S	C		
5		N	N	P	P	P	P	—	—	—	—	—	—	—	—	P	P
15		N	N	P	P	P	P	—	—	—	—	—	—	—	—	P	P
30		N	N	P	P	P	P	—	—	—	—	—	—	—	—	P	P
	1	N	N	N	N	P	P	—	—	—	—	—	—	—	—	P	P
	18	N	N	N	N	N	N	—	—	—	—	—	—	—	—	P	P
	24	—	—	N	N	N	N	N	N	P	P	P	P	P	P	P	P
	48	—	—	N	N	N	N	N	N	P	P	P	P	P	P	P	P

S, *Staphylococcus aureus*, C, *Escherichia coli*, N, no growth, P, growth, —, not done

hours' contact for inhibition of all viable organisms. The oil was found to be more effective against the gram-positive organism *Staphylococcus aureus* (10<sup>-4</sup>) than against the gram-negative organism *Escherichia coli* (10<sup>-3</sup>) after 24- and 48-hour contact periods. No lethal effect was evidenced against either micro-organism by the solvent mineral oil alone.

The oil was found to have a low surface tension and a high spreading ability. On the surface of a seeded agar plate 1 to 2 drops of the oil were observed to spread over several square cm of the agar surface. To permit measurable results the agar well technique was devised (see Methods).

Table 2 depicts the antibiotic effect of the two oil fractions separated from the root of *Leptotaenia dissecta* against 41 strains and species of bacteria and 21 strains and species of molds and fungi. Both fractions were more effective against gram-positive than against gram-negative organisms. Oil fraction 657B33 obtained in the later stages of the steam distillation showed little or no effect on several gram-negative bacteria but was observed to inhibit the majority of gram-positive bacteria.

TABLE 2  
Antibiotic activity of extracts

ORGANISM	INHIBITION IN MM	
	Oil 657B33	Oil 657B30
<i>Shigella paradysenteriae</i>	CPFD	P17
<i>Shigella dysenteriae</i>	N	C20×15
<i>Shigella sonnei</i>	PAE	C16×18
<i>Eberthella typhosa</i>	PAE	C20×22
<i>Pseudomonas fluorescens</i>	PAE	C5×11
<i>Pseudomonas aeruginosa</i>	N	C12×14
<i>Proteus</i> sp	N	C20×22
<i>Proteus vulgaris</i>	N	C18×20
<i>Bacillus circulans</i>	CGD	CED
<i>Escherichia coli</i> 0	PAE	C21×25
<i>Escherichia coli</i> 1	PAE	C21×24
<i>Escherichia coli</i> 2	PAE	—
<i>Escherichia coli</i> S A W	N	C16×20
<i>Staphylococcus aureus</i>	PAE	C14×17
<i>Staphylococcus aureus</i> 1	PAE	C16×20
<i>Staphylococcus aureus</i> 2	PAE	C18×20
<i>Streptococcus faecalis</i>	PAE	C29
<i>Streptococcus viridans</i> J	CS	CP20
<i>Streptococcus viridans</i>	C10	C16
<i>Streptococcus pyogenes</i>	—	C24
<i>Diplococcus pneumoniae</i> type 18	C25	CED
" <i>Afucosus capsulatus</i> "	C25	C28
<i>Micrococcus tetragenus</i>	—	C18
<i>Corynebacterium diphtheriae</i>	CAE	C14×18
<i>Hemophilus influenzae</i>	—	C90
<i>Neisseria intracellularis</i>	—	PAE
<i>Neisseria gonorrhoeae</i>	—	PAE
<i>Bacillus subtilis</i>	CPGD	C20×25 P22×23
<i>Bacillus megatherium</i>	PGD	CPED
<i>Mycobacterium phlei</i>	C17	C18
<i>Mycobacterium smegmatis</i>	C15	C25
<i>Mycobacterium tuberculosis</i> H37	—	CED
" <i>Zooglear</i> " sp	CED	C38
<i>Serratia marcescens</i>	N	CP20
<i>Achromobacter lacticum</i>	PAE	CP15×18
<i>Clostridium putrificum</i>	—	C*
<i>Clostridium histolyticum</i>	—	P*
<i>Clostridium perfringens</i>	—	P*
<i>Clostridium botulinum</i>	—	P*
<i>Clostridium sporogenes</i>	—	P*
<i>Agrobacterium</i> sp	CPED	C20
<i>Mycoderma lactis</i>	CPGD	CED
<i>Mycoderma</i> sp	PAE	C14
<i>Penicillium</i> (Waksman)	PAE	CED

Legend C—complete, P—partial, S—stimulation, FD—fair diffusion, GD—good diffusion, ED—excellent diffusion, AE—area exposed, N—negative

\* Broth

TABLE 2—Continued

ORGANISM	INHIBITION IN MM	
	Oil 657B33	Oil 657B30
<i>Penicillium cyclopium</i>	CAE	CED
<i>Aspergillus niger</i>	PAE	CPGD
<i>Aspergillus terreus</i>	PAE	CPGD
<i>Streptomyces griseus</i>	CED	—
<i>Trychophyton</i> sp	CPED	CED
<i>Trychophyton</i> sp	CPED	CED
<i>Trychophyton sulphureum</i>	—	C18
<i>Candida albicans</i>	PAE	PED
<i>Microsporium trichoderma</i>	PAE	CED
<i>Pythium debaryanum</i>	PAE	C18×18
<i>Mucor sylvaticus</i>	C11P20	C17
<i>Fusarium culmorum</i>	PAE	C36P39
<i>Fusarium</i> sp 2	PAE	PFD
<i>Fusarium</i> sp 3	PAE	C30
<i>Rhizoctonia solani</i>	CPGD	CPFD
<i>Rhizoctonia oryzae</i>	CPAE	PAE
<i>Pestalotia funera</i>	C20	C18
<i>Coccidioides immitis</i>	—	CED
<i>Histoplasmosis capsulatum</i>	—	CED

To facilitate interpretation of the symbols used in table 2 the following explanation is presented. *Shigella sonne* was partially inhibited in the area exposed (PAE) by 657B33, with complete inhibition in an area of 16 by 18 mm (C16 × 18) by 657B30. *Bacillus megatherium* showed complete to partial inhibition with good diffusion using 657B33 (CPGD) and complete to partial inhibition with excellent diffusion using 657B30 (CPED). Complete inhibition was observed at the central portions of the affected area surrounded by gradual partial inhibition.

Attempts were made to ascertain the diffusion of the active agent with a minimum amount of "creeping." The agar well technique was found to minimize this action. With three microorganisms, *Hemophilus influenza*, *Coccidioides immitis*, and *Histoplasmosis capsulatum*, growth was inhibited by fraction 657B30 in the entire plate area (90 mm). It was thought that "creeping" action of the oil might account for some of this diffusion, with a possible thin film of oil spreading over the surface of the agar. However, plates seeded with *Bacillus circulans*, *Bacillus megatherium*, and *Microsporium trichoderma* were inhibited in one-half to three-fourths of the area of the petri plate (45 to 60 mm).

Fraction 657B33 was only partially effective against three strains of *Escherichia coli*. The fourth strain of this species was not affected by 657B33, but 657B30 completely inhibited the growth in an area of good diffusion. The enteric organisms *Eberthella typhosa*, *Shigella paradysenteriae*, *Shigella dysenteriae*, and *Shigella sonne* were completely inhibited by the 657B30 fraction. The oil fraction 657B33 was much less effective in inhibiting these same strains. Fraction

657B33 appeared to be without significant action upon *Pseudomonas aeruginosa*, *Proteus* sp, and *Proteus vulgaris* and had only partial action on *Pseudomonas fluorescens*. These same organisms were observed to be highly susceptible to the action of fraction 657B30. The gram-negative cocci *Neisseria intracellularis* and *Neisseria gonorrhoeae* were only partially inhibited by oil fraction 657B30. These two microorganisms were the only gram-negative bacteria that showed any degree of resistance to fraction 657B30.

Both oil fractions exhibited marked bacteriostatic and bactericidal activity against gram-positive bacteria. The exceptions to this were a slight effect of fraction 657B33 against the three strains of *Staphylococcus aureus* and *Streptococcus faecalis*. Fraction 657B30 was observed to be highly effective against these microorganisms. The fractions inhibited the growth of several human pathogens of gram-positive coccal types, *Streptococcus viridans* (2 strains), *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and "*Mucosus capsulatus*".

Fraction 657B30 was tested against species of *Clostridium* the effective agent in this oil completely inhibited the growth of *C. putrificum*, but was only partially effective in controlling the growth of *C. histolyticus*, *C. perfringes*, and *C. sporogenes*. Two nonpathogenic species of *Mycobacterium*, *M. phlei* and *M. smegmatis*, and one pathogenic strain, *M. tuberculosis* H37, exhibited marked susceptibility to the action of the oil fractions.

Fraction 657B30 exhibited fungistatic activity toward *Penicillium* (Waksman), *Penicillium cyclopium*, *Aspergillus niger*, and *Aspergillus terreus*. Fraction 657B33 exhibited only a partial effect against these organisms but was found to be highly effective against *Streptomyces griseus*. *Candida albicans* was observed to be partially inhibited by the oil fractions. Other representatives of the human dermatophytes, species of *Mycoderma*, *Trychophyton*, *Microsporum*, and *Mucor*, exhibited marked susceptibility to both agents. Two species of phytopathogens, *Fusarium* sp 2 and *Rhizoctonia oryzae*, were observed to be partially resistant to both fractions. Other members of this group, *Fusarium culmorum*, *Fusarium* sp 3, *Rhizoctonia solani*, and *Pestalotia funera*, were susceptible to the effective agent of fraction 657B30.

**Toxicity tests** No deaths occurred when the oil fraction diluted 1:100 in sterile mineral oil was injected intraperitoneally in 0.25- and 0.5-ml amounts, the only toxic sign observed was a staggering gait for periods up to 30 minutes after the injections. A 1:3 dilution of 657B30 fraction intraperitoneally administered in 0.25-ml doses proved lethal in 45 minutes to one animal, the remainder succumbed in 24 to 48 hours. Of six mice receiving the inoculated oil subcutaneously (0.25 ml) one mouse died in 18 hours, the remaining animals tolerated a second injection 48 hours later. Mineral oil alone had no effect on test animals.

#### DISCUSSION

The active agent or agents of the oil fractions were observed to inhibit in varying degrees the growth of all microorganisms tested. The inhibition zones observed were not due to the oil per se as definite areas of diffusion were noted

Excessive diffusion on several test plates could possibly have been due to a thin microscopic film of the oil creeping over a portion of the agar. Even allowing for this possibility, diffusion of the active principal into the agar and out into the periphery of the test areas was much greater than is usually observed with other antibiotic agents, such as penicillin and streptomycin. No local sensitization was observed by the authors from handling these agents over a period of several months.

#### SUMMARY

The antibiotic activity of oil fractions separated from the root of *Leptotaenia dissecta* was determined on 62 strains and species of bacteria, molds, and fungi. The heat-stable active agent was bactericidal for gram-positive bacteria at  $10^{-4}$  dilution and at  $10^{-3}$  dilution for gram-negative bacteria. Fraction 657B30 was found to be of low toxicity for mice as well as highly effective against various fungi and molds.

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# THE PROBLEM OF "DORMANCY" IN BACTERIAL SPORES<sup>1</sup>

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Our experience with the germination of spores of various species of clostridia, especially *Clostridium botulinum*, has led to conclusions that we believe warrant the consideration of investigators of this group of organisms and of the aerobic sporeformers as well. Pertinent observations have been made by previous authors, but it appears that all the recent available evidence on, and various aspects of, the dormancy problem have not hitherto been crystallized into one comprehensive concept.

The essential points already established (Morrison and Rettger, 1930*a,b*, for review of previous literature, Curran and Evans, 1937, Olsen and Scott, 1946, Wynne and Foster, 1948*a*, Foster and Wynne, 1948) are these:

(1) Colony counts from a spore suspension may be submaximum in a few days' incubation, and increase with time over prolonged periods. This indicates delayed germination and is a well-known phenomenon for both aerobic and anaerobic spores. This is the characteristic feature of classical dormancy.

(2) The effect depends on the medium used, being marked in some and non-existent in others.

(3) The final spore counts of a given suspension depend greatly upon the medium employed, though in each medium counts are at the maximum obtainable in it.

(4) Certain substances, notably soluble starch (0.1 per cent), eliminate the delayed germination in some media. In others the starch increases the final maximum count, whereas in still others it has no apparent effect. In general, all complex media preferred for counting spores show the starch effect.

Data exemplifying all these points may be found in table 1. From the information in the literature, as well as in table 1, the following points may be made:

(a) The so-called "dormancy" in these organisms is not an inherent function of the spores, but rather of the medium in which the spores are placed to germinate.

(b) Even when "dormancy" occurred, or in media with low final counts, the large size of colonies speaks against a nutritional inadequacy.

<sup>1</sup> The research which this paper reports was undertaken in co operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or having the endorsement of the War Department.

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(c) Accordingly, a plausible conclusion is that "dormancy," i.e., delayed germination, and also submaximal counts are due to substances in normal media inhibitory to spore germination. "Dormancy" would then be only a manifestation of antigermiation substances in the medium and seemingly is, therefore, merely an artifact.

(d) In the case of the characteristic delayed rate of spore germination during "dormancy," one may consider this to be a function of subinhibitory concentrations of certain substances in the normal medium.

TABLE 1

*C. botulinum* 62A spore recoveries in various agar media\*

BASAL MEDIUM	1 PER CENT SOLUBLE STARCH	INCUBATION, DAYS			
		3	6	17	33
Yesair's plus 0.1% starch	—	45	41	36†	—
Difco nutrient	—	28	30	22	—
Difco nutrient	+	35	42	35	—
Difco brain heart infusion	—	4	6	6	—
Difco brain heart infusion	+	12	23	30	30
BBL anaerobic	—	0	0	2	—
BBL anaerobic	+	0	1	6	8
Difco yeast extract, 1.0%	—	19	17	15	—
Difco yeast extract, 1.0%	+	15	12	11	—
Corn steep liquor solids, 1.0%	—	8	10	8	—
Corn steep liquor solids, 1.0%	+	7	9	11	14
Fresh beef infusion†	—	43	50	17	—
Fresh beef infusion†	+	60	60	53	—
Fresh liver infusion†	—	24	24	23	—
Fresh liver infusion†	+	46	52	49	—

\* All media contained 0.2 per cent glucose and 0.2 per cent thioglycolate, initial pH 7.2 to 7.4.

† The somewhat lower counts in this column as compared to the 6 day values are due to the obscuring of small colonies by the development of gas bubbles and cloudiness in the media.

‡ Prepared exactly like Yesair's medium (cf Wynne and Foster, 1948).

(e) In the case of submaximal counts, the inhibitory substances are of such a nature and concentration that the germination rate of a portion of the spore population is zero, i.e., they never germinate, for all practical purposes.

(f) The inhibitors evidently are of different types, but one is the  $C_{18}$  unsaturated fatty acids (oleic, linoleic, etc.), which are present in almost all complex natural media. The addition of these substances to media suppresses the spore germination rate of clostridia in a manner reminiscent of "dormancy."

(g) The appreciable increase in germination rate, occasioned by the soluble starch in some media, is due to absorption of certain inhibitory substances, including the  $C_{18}$  fatty acids. In some media starch has little or no effect in enhancing counts known to be low.

(h) The different degrees of inactivation of germination inhibitors by starch indicate that different media contain varying amounts of different germination inhibitors. Solvent extraction of some media indicates that some of these naturally occurring inhibitors are nonlipoid.

(i) The significance of these substances inhibitory to germination is evidenced by the fact that the fresh infusions of beef, pork, and liver, ordinarily considered ideal nutrients, contain significant amounts of germination inhibitors, as demonstrated by the considerable enhancement of germination caused by the addition of starch. Incidentally, fresh beef or liver infusion with starch is just as suitable as the widely used (for anaerobes) pork infusion medium of Yesair, with added starch.

(j) Effects similar to that of starch have been observed in the past when supplements containing native proteins and polysaccharides were added to the usual bacteriological media. Though this action has been arbitrarily ascribed to unspecified nutritional factors, an equally plausible idea is that such substances function physically in the manner of starch, through colloid absorption of inhibitory substances, and in some cases this has been proved definitely.

(k) Unless it is shown that no effect is obtained by starch, the foregoing data and ideas make it imperative that this substance be an invariable component of all media in work on anaerobic as well as aerobic spores when maximum counts in the shortest time are desired. Even though starch apparently does not counteract all known cases of retarded germination, it is the simplest over-all treatment available without altering the nutritional status of the basal medium.

(l) The term "dormancy" in the foregoing connection no longer has the degree of validity it formerly enjoyed, and there now appears to be some question as to the justification of its continued use, unless it occurs in cases in which the effect is shown to be independent of the germination medium.

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# INTERFERENCE BETWEEN HUMAN PNEUMONITIS VIRUS AND PSITTACOSIS VIRUS

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In a previous publication (Golub and Wagner, 1948) it was reported that the interference phenomenon could be demonstrated in both eggs and mice with the viruses of meningopneumonitis and psittacosis. Although there is considerable cross reaction between members of the psittacosis-LGV group of viruses as tested by complement fixation (Rake *et al*, 1941), differences in antigenic pattern can be demonstrated to some extent by cross-immunity tests (Beck *et al*, 1944) and by neutralization tests employing hyperimmune rooster serum (Hilleman, 1945, St John and Gordon, 1947). Human pneumonitis virus, S-F strain, has been shown to elicit little or no immunity to psittacosis virus, strain 59, inoculated by the intracerebral route, although some immunity to intra-abdominal challenge by strain no. 59 has been reported (Beck *et al*, 1944, Eaton *et al*, 1941). On the other hand, psittacosis virus will cross-immunize mice to an intracerebral challenge with S-F virus (Beck *et al*, 1944), suggesting either a broader antigenic pattern or greater antigenicity of the psittacosis virus.

The procedures in such cross-immunity studies often involve the use of living virus as a vaccine, tending, in many instances, to produce carrier states. This report concerns a study of the interference phenomenon in mice inoculated with living human pneumonitis virus and its relation to the carrier state.

## MATERIALS AND METHODS

**Viruses** The S-F strain of human pneumonitis was originally isolated from the spleen and lungs of two fatal cases of atypical pneumonia (Eaton *et al*, 1941). It has been passed repeatedly through embryonated eggs by the yolk sac route and will kill mice by intracerebral inoculation, but is almost completely ineffective by the intra-abdominal route. The 6BC strain of psittacosis virus was originally isolated by Dr. K. F. Meyer. This strain is lethal in high dilution for mice by both intracerebral and intra-abdominal inoculation.

The virus preparations used were yolk sac suspensions from eggs either moribund or dead following an inoculation of virus by the yolk sac route. The yolk sacs were ground with sterile glass beads, made up to a 10 per cent concentration by weight in sterile nutrient broth, tested for bacterial sterility, and stored in a dry-ice chest.

**$LD_{50}$  titrations** Intracerebral and intra-abdominal titrations were performed by inoculation of 0.03-ml and 0.5-ml volume, respectively, employing 10-fold dilutions in nutrient broth with 8 to 10 mice per dilution. The animals were observed for a period of 21 days and  $LD_{50}$  values were calculated by the method of Reed and Muench (1938).

*Carrier tests* For the determinations of the presence of active virus in mice at various intervals, the animals were sacrificed and the brains or spleens emulsified individually in 5 ml of broth in a Ten Broeck glass grinder. This suspension was inoculated intracerebrally into 5 or 6 mice and deaths were recorded over a period of 21 days. For the purpose of qualitative recording, the following designations were chosen, depending on the number of deaths observed in each group of 5 or 6 mice: 0 or 1 dead, negative; 2 dead, plus-minus; 3 or more dead, positive. Whenever feasible, deaths the specificity of which were in doubt were checked for the presence of virus by microscopical observation of impression smears of the brain stained by Macchiavello's method.

#### EXPERIMENTAL RESULTS

*Interference with intra-abdominal challenge* The differential pathogenicity of the S-F and 6BC virus strains by the intra-abdominal route in mice offers a means of testing interference between these two strains, although only in one direction, i.e., S-F virus as the interfering agent and 6BC virus as the challenge inoculum.

Living S-F virus was inoculated intra-abdominally into a group of mice in a volume of 0.5 ml each. This volume was calculated to contain  $10^{5.3}$  intracerebral  $LD_{50}$  doses. (This procedure results in only an occasional specific virus death.) At intervals of 1 hour and 1, 3, and 6 days groups of these mice were challenged by the intra-abdominal route with the stock 6BC virus in dilutions of  $10^{-6}$ ,  $10^{-7}$ , and  $10^{-8}$ , using 10 mice per dilution. Normal mice of the same age were inoculated at the 1-day interval with the same dilutions of the 6BC virus. The results of this experiment are recorded in table 1.

Five of the S-F-inoculated mice were tested for a carrier state in the spleen on the eighth day according to the procedure described. All of the 5 mice tested were negative.

The results of this experiment indicate an increase in resistance of the test mice to the intra-abdominal inoculation of 6BC virus at each interval tested. The end point of the control titration was not reached, but even if it is assumed that no mice would have died in the next higher dilution, the  $LD_{50}$  would be  $10^{-8.3}$ . This figure is from 1.8 to 2.1 logs higher than any of the titrations in the S-F-inoculated mice and represents a protection, on the average, against approximately 100  $LD_{50}$  doses.

In the next experiment a greater range of intervals was tested, including 6BC inoculations both before and after the S-F inoculation. The same concentration of S-F virus was employed as in the previous experiment. A different stock 6BC virus preparation, having a lower initial titer, was inoculated intra-abdominally in 4 tenfold dilutions, from  $10^{-6}$  through  $10^{-9}$ , employing 8 mice per dilution. The intervals chosen for the 6BC inoculations were (1) 24 hours before the S-F inoculation, (2) 6 hours before, (3) immediately after, and (4) 6 days after. The control titration was performed on normal mice of the same age group at the 6-day interval. The  $LD_{50}$  end points obtained are shown in table 2.

It is apparent from the results that in order to demonstrate any interference

effect between these two viruses by this method the S-F virus, acting as the interfering agent, must be administered at the same time as or before the 6BC virus. The fact that 6 hours' time is sufficient to prevent the operation of the interference effect is evidence of a fairly rapid reaction between infecting virus and host cells.

Carrier tests were performed on 5 mice of the S-F-inoculated group at intervals of 6, 13, and 20 days. At the 6-day interval, 4 out of 5 spleens were positive for active virus, whereas at the 13- and 20-day intervals all were negative.

TABLE 1

*Interference between S-F and 6BC viruses inoculated intra-abdominally in mice*

PRIMARY INOCULATION (INTRA ABDOMINAL)		INTERVAL	SECONDARY INOCULATION (INTRA ABDOMINAL)		MOUSE MORTALITY RATIO (D/T)*	LD <sub>50</sub>
Virus	Do age (IC LD <sub>50</sub> s)		Virus	Dilution		
S-F	10 <sup>6.3</sup>	1 hour	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	6/10 4/10 0/8	10 <sup>-6.5</sup>
S-F	10 <sup>6.3</sup>	1 day	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	6/10 3/9 1/10	10 <sup>-6.5</sup>
S-F	10 <sup>6.3</sup>	3 days	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5/10 3/10 1/10	10 <sup>-6.4</sup>
S-F	10 <sup>6.3</sup>	6 days	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	4/10 4/10 0/9	10 <sup>-6.2</sup>
None	—	1 day	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	10/10 9/10 8/10	10 <sup>-3.3</sup> or higher

\* D/T = Dead mice over total inoculated

At the 20-day interval possible intracerebral cross immunity was tested by inoculating 6BC virus in dilutions from 10<sup>-6</sup> through 10<sup>-9</sup> into S-F-inoculated mice and control mice of the same age. The LD<sub>50</sub> end points were 10<sup>-6.8</sup> and 10<sup>-7.6</sup>, respectively. These results confirm previous statements in the literature (Beck *et al.*, 1944) on the lack of a strong intracerebral cross immunity to 6BC virus when S-F virus is used as the immunizing agent.

*Interference with intracerebral challenge.* Preliminary carrier tests on the brains of several mice, previously inoculated intra-abdominally with living S-F virus, had indicated that active virus could be recovered from this region for at least several days. It was felt that the presence of active S-F virus might be playing a role in whatever intracerebral resistance to 6BC virus was demon-

strable In order to test this possibility, a group of mice were inoculated by the intra-abdominal route with  $10^{6.3}$  intracerebral  $LD_{50}$  doses of active S-F virus. At intervals of 1, 3, 10, and 21 days thereafter, intracerebral resistance to 6BC virus was tested by inoculation of stock virus in dilutions from  $10^{-6}$  through  $10^{-9}$ , using 8 mice per dilution. A control titration was run on normal mice at the 10-day interval. On the same days as the groups of mice were tested for intra-

TABLE 2

*The effect of sequence and time intervals of inoculations on interference between S-F and 6BC viruses*

INTERFERING AGENT (INTRA ABDOMINAL)		INTERVAL*	SECONDARY INOCULATION (INTRA ABDOMINAL)		MOUSE MORTALITY RATIO (D/T)	LD <sub>50</sub>
Virus	Dosage (IC LD <sub>50</sub> 's)		Virus	Dilution		
S-F	$10^{6.3}$	-24 hours	6BC	$10^{-6}$	7/8	$10^{-7.5}$
				$10^{-7}$	7/8	
				$10^{-8}$	1/8	
				$10^{-9}$	1/8	
S-F	$10^{6.3}$	-6 hours	6BC	$10^{-6}$	8/8	$10^{-7.5}$
				$10^{-7}$	5/8	
				$10^{-8}$	2/8	
				$10^{-9}$	1/7	
S-F	$10^{6.3}$	0 hours	6BC	$10^{-6}$	6/8	$10^{-6.3}$
				$10^{-7}$	0/8	
				$10^{-8}$	0/8	
				$10^{-9}$	0/8	
S-F	$10^{6.3}$	+6 days	6BC	$10^{-6}$	2/8	$10^{-6.8}$
				$10^{-7}$	4/8	
				$10^{-8}$	0/6	
				$10^{-9}$	0/8	
None	—	+6 days	6BC	$10^{-6}$	5/8	$10^{-7.4}$
				$10^{-7}$	5/8	
				$10^{-8}$	3/8	
				$10^{-9}$	2/8	

\* Based on the time of inoculation of the S-F virus

cerebral resistance, carrier tests were performed on both brains and spleens of 5 mice. The results are presented in table 3.

By the tenth day after inoculation with S-F virus there appeared again slight increased resistance to intracerebral challenge with 6BC virus (one log difference in  $LD_{50}$  end point). The group tested on the twenty-first day showed approximately the same degree of resistance. With regard to the carrier studies, it is apparent that active virus reaches the brain in demonstrable concentration by the third day and remains in some of the animals through 10 days. By the twenty-



first day only 1 of 5 mice showed active virus in the brain. The results with the spleen tests suggest that the carrier state of S-F virus in this organ is even more transitory than in the brain, no active virus being recovered on the tenth day. When the results of the 6BC challenge are considered together with the results of the carrier tests on the brain, it would appear that the increased intracerebral resistance begins to manifest itself at a time when the S-F virus concentration in the brain is decreasing. On the other hand, at the 3-day interval, when

TABLE 3  
*Intracerebral resistance to 6BC virus following intra-abdominal inoculation of S-F virus*

PRIMARY INOCULATION (INTRA ABDOMINAL)		INTER VAL	RESULTS OF CARRIER TESTS		SECONDARY INOCULATION (INTRA CEREBRAL)		MOUSE MORTALITY RATIO (D/T)	LD <sub>50</sub>
Virus	Dosage (IC LD <sub>50</sub> 's)		Brain	Spleen	Virus	Dilution		
S-F	10 <sup>6.3</sup>	1 days	-, -, -, -, -	-, -, -, +, +	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	8/8 5/8 3/8	10 <sup>-7.5</sup>
S-F	10 <sup>6.3</sup>	3	+, +, +, +, +	±, ±, +, +, +	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	8/8 5/7 2/8	10 <sup>-7.5</sup>
S-F	10 <sup>6.3</sup>	10	-, -, +, +, +	-, -, -, -, -	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	5/8 3/7 0/7	10 <sup>-8.5</sup>
S-F	10 <sup>6.3</sup>	21	-, -, -, -, +	-, -, -, -, -	6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	6/8 1/6 0/7	10 <sup>-8.4</sup>
None	—	10			6BC	10 <sup>-6</sup> 10 <sup>-7</sup> 10 <sup>-8</sup>	8/8 7/8 1/7	10 <sup>-7.5</sup>

all the mice tested showed definite evidence of active S-F virus in the brain, no intracerebral resistance was found. These results indicate that increased resistance cannot be accounted for by the presence of living S-F virus in the brain, although it may be the result of a carry-over cellular effect.

In this regard, Vilches and Hirst (1947) have reported in a recent publication that PR8 influenza virus inoculated intracerebrally into mice results in a marked interfering effect against WEE virus. The full interference activity was demonstrable for a period of 7 days following the primary inoculation and gradually declined thereafter. Tests on the persistence of the PR8 virus in the brains of mice thus inoculated showed that no growth of the virus took place, but rather

that a consistent decrease in infective concentration was observed until, by the fifth day, no activity was demonstrable in a  $10^{-1}$  dilution

In the case of the S-F virus, however, there is indication of growth in the spleen and in the brain following intra-abdominal inoculation in high concentration, although the infection is only infrequently lethal by that route. In spite of this, intracerebral resistance was not evident at the period when interference would be expected

When it was found that intra-abdominal resistance and intracerebral resistance persisted beyond the period at which the virus appeared most consistently in the organs tested, an attempt was made to differentiate interference effects from cross immunity by means of the time of appearance of antibodies in the blood stream of the animals. Accordingly, groups of 10 mice were bled at intervals of 0, 3, 6, 12, and 24 days after receiving an intra-abdominal inoculation of living S-F virus, as in the experiments just described. The sera from each pooled group was inactivated at 56 C for 30 minutes. Complement-fixation tests were run using a 1:4 dilution of a phenolized antigen prepared from S-F-infected yolk sacs. An S-F-immune rabbit serum was included among the controls.

None of the mouse sera showed a positive complement-fixing reaction in the lowest dilution tested (1:4). The positive rabbit serum control showed four-plus fixation through a dilution of 1:16.

When an increased resistance is demonstrated in the animals within a few days of the primary inoculation, there is little doubt that the primary factor is the interference effect. When the time intervals between the interfering and challenge inoculations are increased, however, the question arises, in cases where the agents involved are antigenically related, as to when interference ceases and immunity begins. The complement-fixation tests described above shed no light on this matter, since the antibodies, which would be evidence of antigenic immunity developing, did not appear.

The following experiment was performed in order to investigate possible intracerebral interference between these two viruses under conditions in which the interfering virus could be inoculated directly into the brain in known concentration. Since both viruses involved are lethal by this route, preliminary immunization to the S-F virus was required. One group of mice were inoculated subcutaneously with 0.5 ml of a  $10^{-3}$  broth dilution of S-F virus (representing approximately  $10^{3.5}$  intracerebral  $LD_{50}$  doses) as the immunizing injection, and a control group were inoculated subcutaneously with 0.5 ml of sterile broth. Fourteen days later the two groups were divided and reinoculated intracerebrally in a volume of 0.03 ml as follows:

GROUP NO	IMMUNIZING OR CONTROL INOCULATION (SUBCUTANEOUSLY)	INTERFERING OR CONTROL INOCULATION (INTRACEREBRALLY)
1	S-F virus	S-F virus ( $10^{3.5}$ $LD_{50}$ doses)
2	S-F virus	Sterile broth
3	Sterile broth	Sterile broth
4	Sterile broth	S-F virus ( $10^{4.5}$ $LD_{50}$ doses)

Two days later, groups 1, 2, and 3 were challenged with 6BC virus, intracerebrally or intra-abdominally, in dilutions from  $10^{-6}$  through  $10^{-9}$ , using 8 mice per dilution. Group no 4 remained as controls. The results of these titrations are presented in table 4.

TABLE 4  
*Resistance of mice to 6BC virus following subcutaneous immunization and intracerebral inoculation with S-F virus*

GROUP NUMBER	IMMUNIZING INOCULATION (SUBCUTANEOUS)	INTERFERING INOCULATION (INTRACEREBRAL)	CHALLENGE INOCULATION			MOUSE MORTALITY RATIO (D/T)	LD <sub>50</sub>
			Virus	Route	Dilution		
1	S-F	S-F	6BC	IC	$10^{-6}$	7/8	$10^{-6.5}$
					$10^{-7}$	2/6	
					$10^{-8}$	1/8	
					$10^{-9}$	0/8	
				IP	$10^{-6}$	1/8	< $10^{-6.5}$
					$10^{-7}$	1/8	
					$10^{-8}$	0/8	
					$10^{-9}$	0/8	
2	S-F	Broth	6BC	IC	$10^{-6}$	7/7	$10^{-7.5}$
					$10^{-7}$	4/7	
					$10^{-8}$	0/8	
					$10^{-9}$	0/8	
				IP	$10^{-6}$	3/8	$10^{-6.5}$
					$10^{-7}$	4/8	
					$10^{-8}$	2/7	
					$10^{-9}$	0/8	
3	Broth	Broth	6BC	IC	$10^{-6}$	8/8	$10^{-7.5}$
					$10^{-7}$	8/8	
					$10^{-8}$	3/8	
					$10^{-9}$	1/7	
				IP	$10^{-6}$	6/8	$10^{-6.5}$
					$10^{-7}$	7/8	
					$10^{-8}$	6/8	
					$10^{-9}$	2/8	

By comparison of LD<sub>50</sub> values in table 4 it can be seen that both groups no 1 and no 2 showed approximately a 1-log decrease in end point compared to the controls when challenged with 6BC virus by the intracerebral route. These results indicate that the presence of living S-F virus in the brains of the animals in group no 1 did not increase significantly their resistance to the 6BC virus over and above that engendered by the original subcutaneous inoculation of S-F virus. The intra-abdominal resistance of both these groups to the 6BC virus can probably be ascribed to a weak state of cross immunity due to the subcutaneous

S-F inoculation Group no 1, which received S-F virus both subcutaneously and intracerebrally, manifested what is probably greater immunity than that found in group no 2, which had only one S-F injection by the subcutaneous route. This difference may be due to the secondary stimulus offered by the intracerebral injection of S-F virus in group no 1 two days before the 6BC challenge. The mice in control group no 4 all died.

Carrier tests were performed on the brains and spleens of extra mice from group no 1 at intervals of 2, 4, 7, 14, and 28 days (brains only) after the intracerebral inoculation of the S-F virus. Five mice were tested at each time interval. The results showed that in mice immunized and challenged with S-F virus in this manner, the brains continued to harbor active virus throughout the 28 day period tested. At least 4 of 5 mice tested at each interval were positive. The spleens, however, were negative throughout the same period.

This experiment was repeated twice using a smaller original subcutaneous inoculation of S-F virus as the immunizing dose and a small intracerebral inoculation of the same virus as the interfering dose. The mice were challenged with the 6BC virus intracerebrally on the second day following the latter inoculation. The results were very similar to those just described. Carrier tests on the fourteenth day showed virus present in the brains of 4 of 5 mice tested, but again none in the spleens. In the mice receiving only the subcutaneous inoculation of S-F virus, no virus was demonstrable in either brains or spleens when tested at the same interval.

#### DISCUSSION

It is well known that infection with some of the members of the psittacosis-LGV group of viruses results in latent or inapparent infections from which active virus can be recovered for long periods of time (Beck *et al*, 1944, Bedson, 1938). This suggested the possibility that immune reactions, exhibited in animals inoculated with living virus, might be due to the presence of the active virus rather than to a true immunity. The results of the present study, in which interference, immunity, and carrier tests are correlated, do not necessarily support this possibility.

The experiments in which mice were given an inoculation of living S-F virus intra-abdominally and challenged with 6BC virus by the intracerebral route showed that, although active S-F virus was present in the brains within a few days, no interference was evident during this period. Further, the experiments in which S-F-immunized mice were given an interfering dose of S-F virus directly into the brain, followed by an intracerebral challenge with 6BC virus, gave no indication of an increased resistance beyond the cross immunity produced by the S-F immunizing injection.

The results of the tests employing the intra-abdominal route for both interfering and challenge inoculations gave somewhat different results, however, since active virus was recoverable from spleens during the early intervals when an interference effect would be expected. The increased resistance could be demonstrated early and was retained beyond the time when active virus was consist-

ently present. The latter observation might be explained on the basis of a carry-over interference effect, such as was found with influenza and WEE viruses (Vilches and Hirst, 1947), or on the basis of a cross immunity between the two virus strains, or a combination effect. However, no complement-fixing antibodies, even to an homologous antigen, were demonstrable.

When the results of interference experiments performed within the psittacosis group of viruses are compared with observations on several other virus systems studied, it is apparent that a resistance of only a relatively low magnitude is demonstrable within this group. Although the difference in end points between test and control series in several cases was less than 2 logs, when the percentage deaths in the groups were compared, the differences were calculated to be greater than twice the standard error involved.

#### SUMMARY

Interference effects were demonstrated in mice when human pneumonitis virus (S-F strain) was inoculated by the intra-abdominal route followed immediately, or at short intervals, by psittacosis virus (6BC strain) inoculated by the same route. Although active S-F virus reached the brain within a few days under such conditions, the mice did not show an increased intracerebral resistance to psittacosis virus until a longer interval had passed, at which time the S-F virus in the brain was disappearing. The resistance was only slight and due probably to cross immunization.

When human pneumonitis virus was inoculated directly into the brains of immunized mice, no rapid increase in resistance to psittacosis virus by the intracerebral route was observed beyond that engendered by the S-F immunizing injection. These results indicate that the mere presence of living S-F virus in the brain, shown by carrier studies, does not necessarily produce interference effects.

It would appear that cross-immunity studies between these two viruses are not complicated by the interference phenomenon when the intracerebral route is used for challenge. When the intra-abdominal route of challenge is employed, however, a resistance not entirely due to antigenic cross immunity may be found.

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# THE ACTIVATION OF THE BACTERIAL VIRUS T4 BY L-TRYPTOPHAN<sup>1</sup>

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A few years ago I discovered that preparations of the bacterial viruses T4 and T6 produce more plaques when plated with their host, *Escherichia coli* strain B, on Difco nutrient agar (N) than they do on agar containing only inorganic salts and ammonium lactate as a source of nitrogen and carbon (Anderson, 1945b). It, therefore, appeared that some substance or cofactor, present in the nutrient medium, had promoted the activity of these viruses. To identify the activating substance the effect on virus activity of individual growth factors and amino acids was studied. L-Tryptophan was found to be most active in promoting plaque formation by T4, whereas phenylalanine, diiodotyrosine, and tyrosine showed progressively lower activities. Many other synthetic amino acids having aromatic groups are also active, but D-tryptophan appears not to be active (Anderson, 1946a,b). Oddly enough, norleucine is slightly active (Delbruck, 1948).

Regarding the mechanism of action of these substances it was discovered that T4 and T6 particles were not even adsorbed on host cells in the synthetic medium (Anderson, 1945b). This result, first obtained by centrifugation experiments, is strikingly confirmed by electron microscope studies of virus-host mixtures with and without added tryptophan. In the synthetic F medium no T4 particles are seen adsorbed on the host cells (figure 1, no 1), but on the addition of tryptophan to the mixture many tadpole-shaped virus particles may be seen attacking the cell surface (figure 1, nos 2A,B) (Anderson, 1946b). In some way, therefore, L-tryptophan has the power of promoting the *adsorption* of the virus T4 on its host. We shall refer to compounds having such action as *adsorption cofactors*.

Because this phenomenon offers an experimental tool for the study of the specific, but little understood, mechanisms of virus adsorption, it was decided to investigate the matter further—in particular to determine the site of action of the cofactors. This work, which is reported here, shows that it is the virus particles that are activated by L-tryptophan rather than the bacterial cells.

## MATERIAL AND METHODS

The host (*Escherichia coli* strain B), the virus T4, the growth media, and the methods of assay were the same as those reported earlier (Anderson, 1945b).

Virus was grown on the host in 1,500-ml shaker flasks, with aeration at room

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temperature, in the synthetic ammonium lactate medium (F). It was then purified and concentrated by differential centrifugation 1,000 rpm for 20 minutes to remove the unlysed bacteria and 10,000 rpm for 2 hours to sediment the virus. The virus so obtained was resuspended in F medium, and after a few days' or weeks' storage at 4 C the differential centrifugation was repeated. Finally, the material was filtered through a Mandler filter of "medium" porosity to remove bacterial contaminants.

Two such virus concentrates were used in this work. One, designated T4 (5/10/45), was obtained by seeding the shaker flask containing B with about  $10^6$  T4 particles from a clone derived from the original T4 stock. This concentrate assayed about  $1.6 \times 10^{11}$  on nutrient (N) agar and about  $3 \times 10^5$  on F agar. The other T4 concentrate was obtained by seeding a shaker flask containing B with T4r plaque (Hershey, 1946) isolated from an N agar plate used in a routine assay of the T4 (5/10/45) concentrate. This stock concentrate, designated T4r (3/10/47), gave an assay of  $3 \times 10^{11}$  particles per ml on N agar and only  $6 \times 10^5$  on F agar.

Two methods were available for the determination of the relative concentrations of activated virus, T4\*. One method takes advantage of the fact that once the virus particles have become attached to host cells the infected cells are efficient in producing plaques on bacterial smears on F agar at 37 C. The number of plaques observed on this medium is thus taken to be proportional to the concentration (B T4) of bacteria that had been infected under the conditions of adsorption of the virus on the host. The total concentration (T4) of plaque-forming particles will be taken to be proportional to the number of plaques that are formed by appropriate dilutions on Difco nutrient agar (N) or on F agar containing  $10 \mu\text{g}$  L-tryptophan per ml (Anderson, 1945b).

Another method involves the phenomenon of summary lysis (Anderson, 1945a). When the host cells are heavily irradiated with ultraviolet light and then exposed to T4 virus in the presence of L-tryptophan, they are rapidly lysed without the multiplication of the virus. The rate and extent of lysis was found to be a qualitative measure of the amount of T4\* in the mixture of virus and irradiated B.

FIGURE 1

*Procedure* *E. coli* strain B grown for 18 hours at 20 C on a synthetic medium (F) slant was suspended in 1 ml of liquid F medium, and 0.1 ml samples were placed in test tubes numbered 1 and 2. One-tenth ml of T4 virus suspension in synthetic (F) medium was added to test tube no. 1 and 0.1 ml of T4 virus in synthetic (F) medium containing  $100 \mu\text{g}$  L-tryptophan per ml was added to test tube no. 2. The tubes stood 3 minutes at 25 C. A droplet from each test tube was then placed on a separate formvar membrane. After the membranes had stood 30 seconds, they were washed 8 times in distilled water and allowed to dry. The specimens were then placed in the electron microscope holders, shadowed with gold (Anderson, 1946c), and examined in an RCA type B electron microscope with no limiting objective aperture.

No. 1 *E. coli* B + T4 virus from test tube no. 1 containing no added cofactor. No adsorbed virus is visible. EMG 213d  $\times 10,000$ .

No. 2A *E. coli* B + T4 virus from tube no. 2 containing  $50 \mu\text{g}$  L-tryptophan per ml. Many virus particles are adsorbed on each host cell. EMG 216d  $\times 10,000$ .

No. 2B *E. coli* B + T4 virus from tube no. 2. EMG 217d  $\times 25,000$ .





FIGURE 1

## EXPERIMENTAL RESULTS

*The Dependence of T<sub>4</sub> Activity on Tryptophan Concentration*

*Summary of lysis method* To each of 6 colorimeter tubes was added 0.1 ml of the concentrated suspension of T<sub>4</sub> (5/10/45). To each was then added 0.1 ml of F medium containing 14, 10, 6, 4, 2, and 0  $\mu$ g of DL-tryptophan, respectively. About 5 minutes later, at time  $t = 0$ , to the first tube were added 1.0 ml of a suspension of B that had previously been irradiated with ultraviolet light from

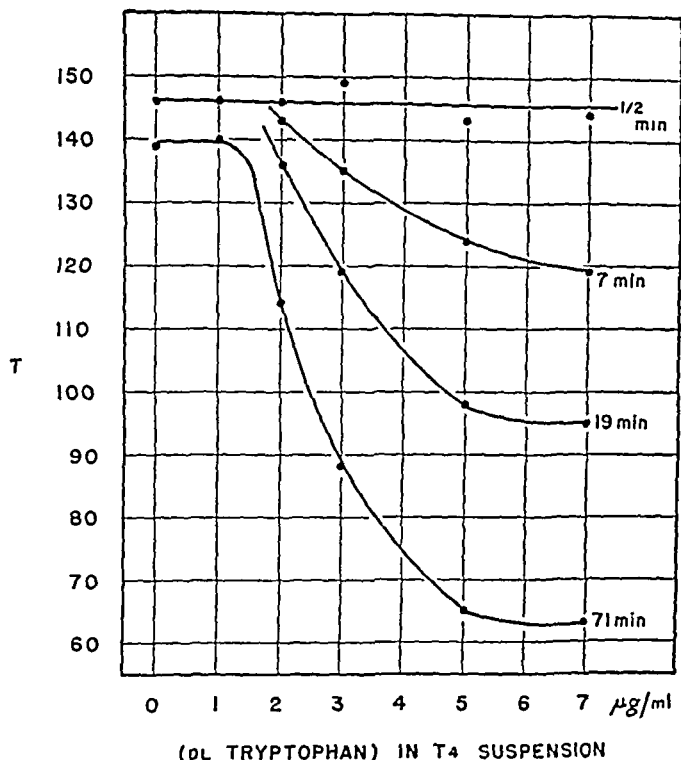


FIG. 2. THE LYSIS OF IRRADIATED B SUSPENSIONS ADDED TO T<sub>4</sub> (5/10/15) CONCENTRATIONS CONTAINING VARIOUS ADDED CONCENTRATIONS OF L TRYPTOPHAN.

The turbidities,  $T$ , of the mixtures of bacteria and virus are plotted against the tryptophan concentrations with which the T<sub>4</sub> was incubated.

an H-4 lamp (Anderson, 1945a). At  $t = \frac{1}{2}$  minute the turbidity of this suspension was read with a Klett colorimeter. At  $t = 1$  minute 4 ml of the irradiated B suspension were added to the second tube, its turbidity was read at  $t \approx 1\frac{1}{2}$  minutes, and so on until irradiated B had been added to each of the tubes and the turbidities of all the mixtures determined. At later intervals the suspensions were taken in turn, shaken gently to prevent settling of the bacteria, and their turbidities read. The results are presented in figure 2, where the turbidity readings are plotted as ordinates against the concentration of tryptophan in the virus suspension before the addition of the bacteria. Curves have been drawn

through the points for each of the various time intervals. It is seen that the measurable lytic activity increases rather abruptly from nil for virus exposed to less than about 1.5  $\mu\text{g}$  DL-tryptophan per ml to a marked value at 2  $\mu\text{g}$  per ml.

In another experiment it was found that no measurable lysis was induced by adding the same total amounts of tryptophan to 4-ml mixtures of virus and irradiated B. These results indicate that the tryptophan activated the virus in the concentrated suspensions, rather than the bacteria after they were mixed with the T4-tryptophan suspensions.

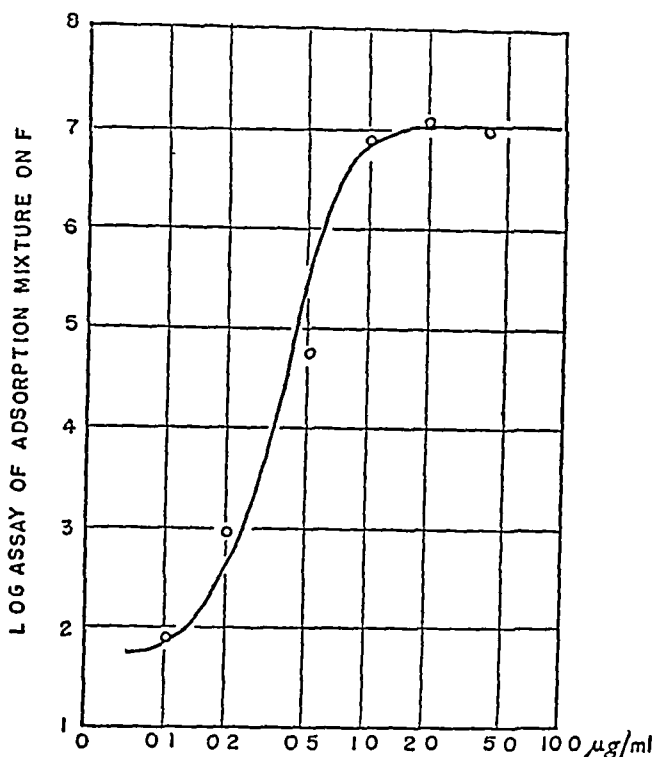
*Plaque count method.* In a series of preliminary experiments samples of T4 (5/10/45) were incubated with L-tryptophan at various concentrations in F. Ten ml of B in F were then added with stirring to 0.1-ml samples of the T4-tryptophan mixtures. After 5 minutes had been allowed for adsorption, the mixtures were assayed for (B T4) and for (T4) on F and N agar, respectively. The ratios of the assays on F to the assays on N rose 100-fold for incubation concentrations between 0.2 and 10  $\mu\text{g}$  L-tryptophan per ml. In parallel experiments in which L-tryptophan was added to mixtures of T4 and B in F, a corresponding rise in (B T4) did not occur until the tryptophan concentrations in the mixtures were brought to 0.2 to 10  $\mu\text{g}$  L-tryptophan per ml. Since the concentrations to which the B mixtures were exposed in the first experiments were only 1/100 of these values, whereas those to which the virus had been exposed were the same, it appeared that the tryptophan acted on T4 in making it capable of adsorption rather than on B in making it "receptive" to the virus.

An experiment in which a series of T4r (3/10/47) samples were incubated with various tryptophan concentrations and adsorbed on B at the same tryptophan concentrations is summarized in figure 3. It is seen that the number of plaques obtained on F agar increased over 100,000-fold as the tryptophan concentrations with which the T4 was incubated increased from 0.1 to 10  $\mu\text{g}$  per ml. Since the only systematic variable in the experiment was the tryptophan concentration to which the virus had been exposed we may conclude that the effect of tryptophan was on the virus.

#### *Activation of T4 by L-Tryptophan as a Function of Temperature*

*Summary of lysis method.* Samples of T4 (5/10/45) were exposed to 6  $\mu\text{g}$  DL-tryptophan per ml for various lengths of time at room temperature and at 0 C. Irradiated host bacteria were then added to each in turn and the turbidities of each tube read at intervals. The results, plotted in figure 4, show that the temperature of exposure of the virus to this cofactor had a decided effect on the virus activity, little or no activity resulting from exposure at 0 C and marked activity resulting from analogous exposures at room temperature.

*Plaque count method.* In three experiments, 0.1-ml samples of T4 (5/10/45) containing 20  $\mu\text{g}$  of L-tryptophan per ml were placed in test tubes and incubated at various temperatures for 1½ to 5 hours. To them were then added with stirring 10-ml volumes of actively growing B in F medium at 37 C. The mixtures were then assayed on F agar for (B T4) and on N agar on F agar containing 10



CONCENTRATION OF L-TRYPTOPHAN IN ACTIVATION TUBE

FIG. 3. THE FORMATION OF PLAQUES ON F MEDIUM BY MIXTURES OF HOST BACTERIA AND T<sub>4</sub> THAT HAD BEEN ACTIVATED BY VARIOUS CONCENTRATIONS OF L-TRYPTOPHAN

*Procedure.* T<sub>4</sub>r (3/10/47) was diluted 1:10 in F medium containing 100 μg of L-tryptophan. Various dilutions of this mixture were then made and volumes placed in a series of incubation tubes (labeled s to y) such that each tube contained 0.2 μg of L-tryptophan and  $6 \times 10^4$  virus particles as follows:

TUBE	s	t	u	v	w	x	y
Volume (ml)	2.0	1.0	0.4	0.2	0.1	0.05	0.5
μg tryptophan per ml	0.1	0.2	0.5	1.0	2.0	4.0	0.1

The incubation tubes were kept at 37°C for 25 minutes, at the end of which time the contents of the following bacterial tubes each containing  $4 \times 10^{10}$  bacteria were added to the corresponding tubes of virus:

TUBE	S	T	U	V	W	X	Y
ml 24-hr-aerated B in F	8	8	8	8	8	8	8
ml F medium	0	1.0	1.6	1.8	1.9	1.95	1.8

After 5 minutes had been allowed for adsorption, the mixtures were assayed on F agar and on N agar for B<sub>T</sub>4 and for total virus, respectively. The assays on N agar were constant, those on F agar varied as shown. Since the conditions in the adsorption tubes were identical, the differences in the assays on F agar must have been due to the differences in the concentration of L-tryptophan with which the virus had been incubated.

$\mu\text{g}$  L-tryptophan per ml for (T4)    The results, expressed as ratios between the adsorbed virus and free virus

$$R = \frac{(B \text{ T4})}{(T4) - (B \text{ T4})}$$

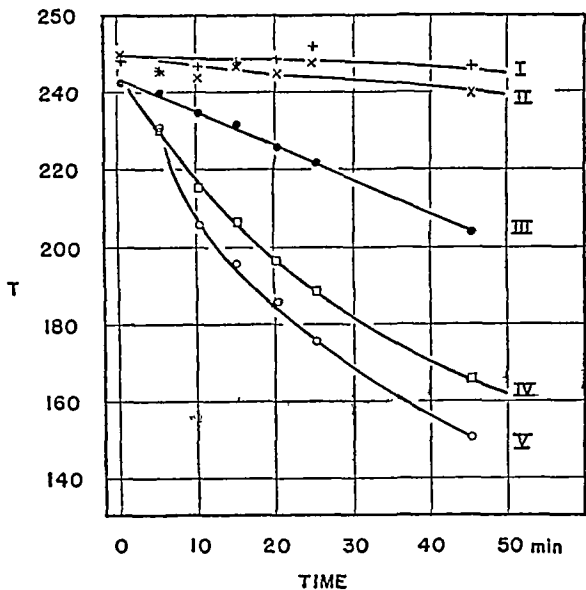


FIG 4 THE EFFECT OF TEMPERATURE OF INCUBATION ON THE ACTIVATION OF T4 (5/10/45) BY 3  $\mu\text{G}$  L-TRYPTOPHAN PER ML AS DETERMINED BY ITS SUMMARY LYSIS OF UV IRRADIATED HOST CELLS

*Procedure* To 0.1 ml samples of T4 (5/10/45) in colorimeter tubes was added 0.1 ml of DL-tryptophan. The tubes were incubated at 0 C and at room temperature as follows

SAMPLE	$\mu\text{G}$ DL-TRYPTOPHAN/ML	SUCCESSIVE TEMPERATURE TREATMENTS		
		Min at room temperature	Min at 0	Min at room temperature
I	0	0	0	10
II	3	0	10	0
III	3	0	5	5
IV	3	5	5	0
V	3	0	0	10

Four samples of B irradiated 10 minutes with the H-4 arc were then added to each and the turbidities (T) read on a Klett colorimeter and plotted against times after mixing

are plotted in figure 5 against the reciprocals of the absolute temperatures T at which the virus was equilibrated with tryptophan

It is seen that the activation is a function of the temperature to which the virus was exposed during contact with cofactor, being a maximum near 35 C and falling off rapidly on either side. The extension of the value to higher temperatures is prohibited by the rather rapid inactivation of the virus above 60 C

*Activation of T<sub>4</sub> by L-Tryptophan as a Function of pH*

*Plaque count method* Aliquots of the T<sub>4</sub> (5/10/45) suspension were added to F medium and, adjusted to various pH's with HCl or NaOH and enough L-tryptophan was added to each to bring the concentration to 20  $\mu$ g per ml. After standing at 28 C for 30 minutes, 0.1-ml samples were added with stirring to 10-ml samples of an aerated culture of B in F medium. The latter mixtures were then assayed for (B T<sub>4</sub>) on F agar and for (T<sub>4</sub>) on F agar containing 10  $\mu$ g of L-tryptophan per ml. The results, expressed in terms of R as before, are plotted in figure 6. It is seen that the activation is again a function of the conditions

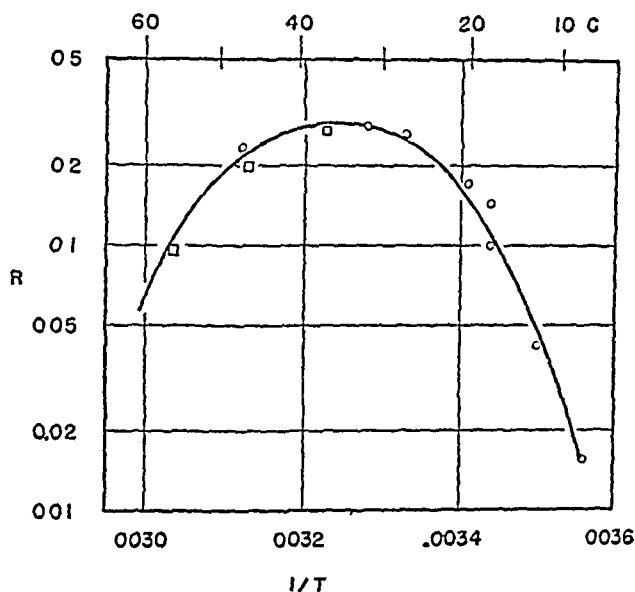


FIG 5 THE ACTIVATION OF T<sub>4</sub> BY 20  $\mu$ G L-TRYPTOPHAN PER ML AT VARIOUS TEMPERATURES

The results of three experiments are plotted. The ratios R of adsorbed to free virus are plotted as ordinates against the reciprocal of the absolute temperature of activation as abscissae.

of exposure of the virus to tryptophan, being a maximum at pH 7.5 and falling off on the acid side. Above pH 8.6 and below pH 3.8 the virus is rapidly inactivated in F medium so the extension of the curve beyond these values is not feasible.

*Activation of T<sub>4</sub> as a Function of the Length of Exposure to L-Tryptophan*

*Plaque count method* T<sub>4</sub> (5/10/45) was diluted 1:50,000 in 200 ml of F medium at 26 C and placed in a Waring-type blender. One-tenth ml from the suspension in the blender was then added to 1.9 ml of F medium that contained  $9 \times 10^3$  B. At time  $t = 0$ , 0.4 mg of L-tryptophan dissolved in 1 ml of F were added to the contents of the blender, and the motor was turned on for 2 seconds to mix the contents. Then at intervals 0.1-ml samples were removed from the blender and added with stirring to 1.9-ml samples of the bacterial suspen-

sions, the time of addition being carefully noted. After 5 minutes had been allowed for adsorption, the B-T4 mixtures were assayed for plaque-forming particles on F and on N agar. The results presented in figure 7 show that the virus rapidly gained activity with the length of time it was exposed to L-tryptophan.

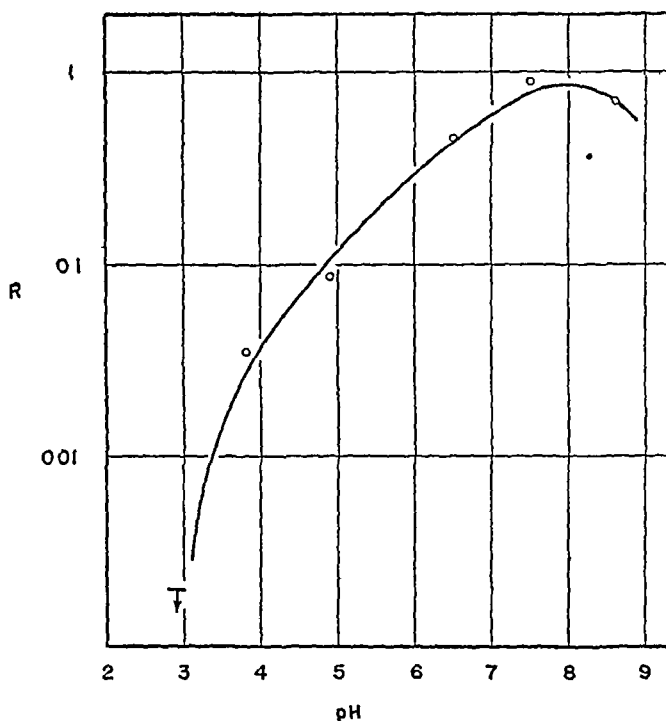


FIG. 6. THE ACTIVATION OF T4 BY 20  $\mu$ G L-TRYPTOPHAN PER ML AT VARIOUS pH'S

The results were expressed as the apparent ratio R between the active to inactive virus and the values plotted above on a logarithmic scale versus the pH. The value R at pH 2.9 was determined to be less than 0.002.

#### *The Deactivation of Tryptophan-activated T4 by Dilution in F Medium*

The results presented so far indicate that the virus T4 is activated by L-tryptophan at concentrations above 0.2  $\mu$ g per ml and that the extent of activation depends on the tryptophan concentration, the temperature, and the pH. A number of observations suggested that this activation is reversible. For example, T4 grown on B in nutrient medium is rapidly adsorbed onto host cells added to the suspension. However, if such a suspension is diluted 1:1,000,000 in F medium and then added to B, only 0.1 per cent of the particles will be adsorbed or form plaques on F agar. The virus must then have lost its activation on dilution in F medium. This rate of deactivation of tryptophan-activated T4 was followed in the following experiment.

One-half cubic centimeter of T4 (5/10/45) was added to 9.5 ml of F medium

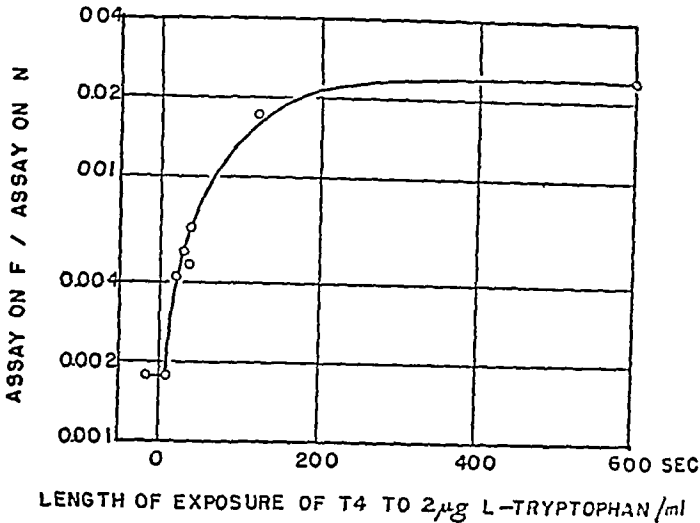


FIG 7 THE ACTIVATION OF T4 (5/10/45) IN F MEDIUM AT 26 C AS A FUNCTION OF THE LENGTH OF EXPOSURE TO 2  $\mu$ g L-TRYPTOPHAN PER ML

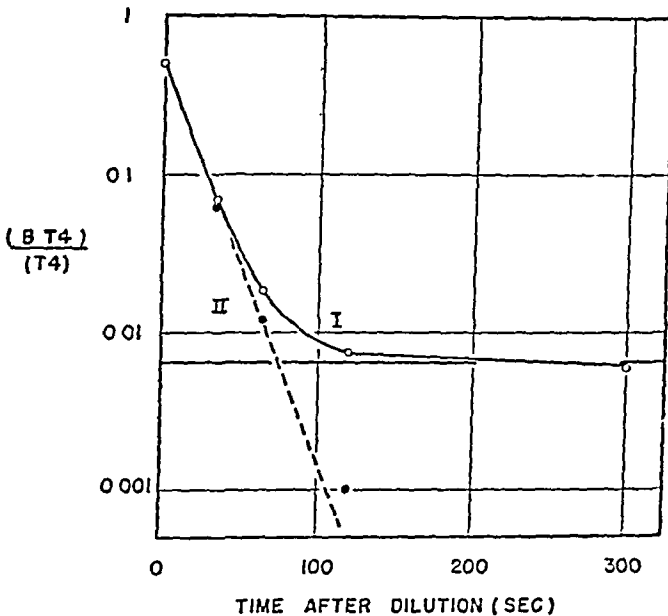


FIG 8 THE LOSS OF ACTIVITY OF T4 ACTIVATED BY 20  $\mu$ g L-TRYPTOPHAN PER ML AFTER DILUTION 1:100 IN F MEDIUM

In curve I the value of (B T4) is seen to drop rapidly and approach the value for virus that had not been exposed to added L-tryptophan. When the latter value is subtracted from the values observed at different times the fractions plotted in curve II were obtained.

containing 20  $\mu$ g L-tryptophan per ml and allowed to stand about 30 minutes at room temperature to activate the virus. At time  $t = 0$ , 0.1 ml of this suspension was added to 10 ml of F medium and stirred rapidly. Since the concentration



of tryptophan was only 0.2  $\mu\text{g}$  per ml in this suspension, the virus might have been expected to lose activity with time. After various time intervals 0.1-ml samples of the diluted suspension were added to 10-ml samples of a suspension of B in F medium. This permitted the virus that had retained its activity to infect host cells to form B T4, which, being capable of forming plaques on F agar, would provide a measure of the decreasing concentration of tryptophan-activated virus in the diluted T4-tryptophan mixture. Appropriate dilutions of the suspensions were then made and samples plated on F agar to provide plaque counts of the total number of plaque-forming particles (T4). The results, shown in figure 8, curve I, show the ratios of infected bacteria to total virus, (B T4) (T4), plotted logarithmically against the time after dilution at which the samples were added to the bacterial suspensions. The point at zero time was obtained by adding 0.1 ml of the T4 in 20  $\mu\text{g}$  L-tryptophan per ml directly to 10 ml of the bacterial suspension and assaying this suspension for infected bacteria (B T4) and total infectious centers (T4). The horizontal line in the graph was obtained by assaying the stock T4 concentrate directly on F and N, making all dilutions in F medium. This quantity represents the fraction of the virus that could form plaques without added cofactor.

It is seen that the concentration of activated T4 in the diluted suspension of tryptophan-activated T4 drops rapidly and then levels off to approach the value for the unactivated suspension. Subtracting the value for the latter suspension from each of the points of curve I, one obtains the dotted curve II, which represents the rate of loss of activity by those T4 particles that had been activated by tryptophan. It is seen that only 2 minutes after dilution all but one 0.1 per cent of the activated virus had lost its activity. The deactivation reaction appears to be first order, but it is so rapid that it is difficult to follow at room temperature with the pipette and test tube techniques employed here. The results of more precise deactivation experiments will be reported shortly. The point to be emphasized here is that the cofactor L-tryptophan activates the virus T4 in a reversible manner.

#### DISCUSSION

A lower limit for the number of tryptophan molecules required for activation may be estimated from the slope of the plot of  $\log (T4^*)$  vs  $\log (\text{tryptophan})$  at equilibrium. Here  $(T4^*)$  represents the concentration of activated T4. Writing the reaction



the law of mass action predicts that at equilibrium

$$\log \frac{(T4^*)}{(T4) - (T4^*)} = K + n \log (\text{L-tryptophan})$$

Since the slope of the activation curve (figure 3) is approximately equal to six we judge that at least six tryptophan molecules must have been involved in the activation of T4. The observed inhomogeneity in the requirements of

the individual particles in the population (Anderson, 1948a) would have the effect of reducing the slope for the population

The activation of T4 by 20  $\mu$ g L-tryptophan per ml as a function of temperature is interesting, like many biological phenomena it displays a maximum at about 35 C. In enzyme systems it is usual to account for activity curves of this sort on the assumption that the reaction rate of the "native" form increases with temperature but that this form is in equilibrium with a nonreactive "denatured" high temperature form whose accumulation at the expense of the native form results in an eventual reduction in rate as the temperature is increased. The explanation of the present results may involve a similar mechanism in which the "receptiveness" of the virus for cofactor is shifted with temperature.

The inactivity of D-tryptophan as compared to L-tryptophan (Anderson, 1946a) suggests a specificity in cofactor phenomena comparable to that observed in enzyme systems. It would seem from the results reported here that, like substrate molecules reversibly bound to enzymes but not reacting until the combination makes liaison with coenzyme or a source of energy, so the cofactor molecules are reversibly bound in the virus structure and do not react chemically until contact with host constituents is made. Rather than a simple, static fitting together of sterically complementary structures of the virus and host, it now seems likely that the adsorption of virus on its host is a dynamic, enzyme-like process. Adsorption may involve enzymatic syntheses (of peptides?) coupled with degradations of the hosts' surface elements.

#### ACKNOWLEDGMENT

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#### SUMMARY

Experiments were performed in which samples of the bacterial virus T4 were exposed to a cofactor for adsorption, L-tryptophan, under various conditions. The samples were then mixed with suspensions of the host, *Escherichia coli* B under identical conditions. It was found that the fractions of virus adsorbed varied with the conditions under which the virus had been exposed to its cofactor.

The percentage activation varied with the following factors: (a) with tryptophan concentration, it was negligible at 0.1  $\mu$ g per ml and rose sharply to high values at 2  $\mu$ g per ml, (b) with temperature, it was maximum at 35 C and fell to low values at 0 C and 60 C, (c) with pH, it was maximum near pH 8, and (d) with the length of exposure (equilibrium with 2  $\mu$ g per ml was practically complete in 2 minutes at 26 C).

When incubated with tryptophan and then diluted in synthetic medium, the virus rapidly lost its ability to be adsorbed on its host. Upon re-exposure to an adequate concentration of a cofactor it regained its ability to be adsorbed.

It is concluded that the cofactor for adsorption, L-tryptophan, activated the bacterial virus T4 in a reversible manner

Possible functions of virus cofactors in the adsorption mechanism are discussed

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# THE INHERITANCE OF REQUIREMENTS FOR ADSORPTION COFACTORS IN THE BACTERIAL VIRUS T4<sup>1</sup>

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Previous work has shown (Anderson, 1945, 1946, and 1948a) that many strains of the bacteriophage T4 are not adsorbed on their host, *Escherichia coli* strain B, unless the virus has been activated by a cofactor such as L-tryptophan. However, all preparations of T4 so far studied contain fractions that form plaques on bacterial smears growing on synthetic medium agar to which no cofactor has been added. The appearance of such plaques suggests that inhomogeneities with respect to cofactor requirements exist in the virus preparations.

The results reported here show that our stocks are, indeed, inhomogeneous with respect to cofactor requirements. The virus particles which form plaques efficiently in the absence of added cofactor can be removed from the stocks by adsorption on the host. Particles that are inefficient in producing plaques without added cofactor are not removed by such treatment. Analyses of individual virus clones arising from particles of different types show that cofactor characters (requirement and nonrequirement) are inherited by members of virus clones.

## MATERIALS AND METHODS

The host for T4, *E. coli* strain B, has been described previously (Anderson, 1945), as has the synthetic ammonium lactate medium (F) and the nutrient medium (N) on which the host was grown in this work. All plates used for assay were incubated at 37 C.

Individual plaques were picked up with medicine droppers equipped with 2-mm tips and rubber bulbs. With the bulb slightly compressed, the tip was pushed through the agar containing the desired plaque and moved a bit to free the agar from the underlying glass of the petri dish. Then, when the bulb was released, atmospheric pressure forced the cylinder of agar containing the plaque into the dropper. The agar cylinder was then ejected into 1 ml of F medium in a small sterile tube. After 20 minutes the fluid was found to contain between  $10^7$  and  $10^9$  virus particles, belonging to a single clone of T4 particles. After incubation at 52 C for 20 minutes, to kill the infected bacteria but not the free virus, the samples were assayed on F agar and N agar, all dilutions being made in liquid F medium.

The original parent strain of T4 had been isolated by Demerec and Fano (1945) from a single plaque or clone appearing on B grown on N agar. These authors

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very kindly gave a sample of their stock preparation to the author. It contained  $2 \times 10^9$  plaque-forming particles per ml on B grown on N agar, and only  $1 \times 10^5$  plaque-forming particles per ml on B grown on F agar. One stock preparation used, T4 (5/10/45), owed its origin to a single plaque isolated from F agar on which B infected with T4 (activated by the cofactors in N medium) had been plated. In subsequent serial passages on F medium an estimated  $10^7$  phage particles would have been formed if all the original particles had multiplied equally. Fifteen hundred ml of F medium containing  $10^{11}$  B in a shaker flask were then seeded with  $7 \times 10^9$  of these phage particles. After aeration overnight, the culture had lysed. The virus was concentrated in the Sharpless supercentrifuge at 45,000 rpm and filtered. The 350 ml of filtered, concentrated virus, T4 (5/10/45), contained  $3.9 \times 10^{11}$  plaque-forming particles per ml on N agar and  $2.6 \times 10^8$  plaque-forming particles per ml on F agar at 37 C. The characters of the virus particles in plaques formed by this stock under various conditions were determined in the initial experiments.

#### RESULTS

*Analysis of individual plaques of T4.* Four plaques were isolated from each of two F and two N plates used in the above assay of T4 (5/10/45). When analyzed, six of the clones obtained from F agar formed almost as many plaques on F agar as on N agar. Two of them gave much lower assays on F agar than they did on N agar. All of the plaques isolated from N agar gave much lower assays on F agar than on N agar (table 1A). It thus appeared that the stock T4 contained at least two types of phage. One required no added cofactor for efficient production of plaques on B on synthetic agar at 37 C. The other type, present at 1,000 times the concentration of the former, was quite inefficient in producing plaques in the absence of added cofactor. Furthermore, it may be noted that plaques like nos. 2 and 3 of table 1, requiring added cofactor for the efficient production of plaques, occasionally appear on synthetic medium at 37 C. Similar plaques were obtained in the assays of cofactor-requiring clones like 5 to 8 and 13 to 16 inclusive.

The question arose: Do these plaques arising on F agar from cofactor-requiring stocks contain predominantly cofactor-requiring particles or not? This was tested by analyzing 20 individual plaques isolated from F and N plates used in the analysis of plaque no. 15. The results given in table 1B show that whether they arise on F or on N agar, clones originating from cofactor-requiring stocks require cofactor.

Do clones arising on N agar from populations such as clone 10, which do not require cofactor, retain this characteristic? Analyses of 10 plaques from F plates and 10 plaques from N plates used in the analysis of clone 10 are given in table 1C. It is seen that the nonrequirement is retained by clones arising on N agar. The cofactor requirements are inherited by T4.

Is L-tryptophan an adequate cofactor for strains arising from T4 (5/10/45)? Fifty-six plaques isolated from T4 (5/10/45) plated on N agar, were assayed on F agar, on F agar containing 20 mg L-tryptophan per liter, and on N agar. The

ratios of assays on F agar to assays on N agar were all less than 0.005. The ratios of assays on F agar plus tryptophan to assays on N agar were all greater than 0.9.

TABLE 1

*Analyses of single plaques formed by particles in T4 (5/10/45) stock*

PLAQUES FROM F PLATES		PLAQUES FROM N PLATES	
Clone no	Ratio Assay on F agar Assay on N agar	Clone no	Ratio Assay on F agar Assay on N agar
A Single plaques from T4 (5/10/45) stock			
1	0.62	5	0.000019
2	0.0004	6	0.000011
3	0.0002	7	0.00006
4	0.83	8	0.000002
9	0.6	13	0.000004
10	1.01	14	0.000002
11	0.9	15	0.0037
12	0.9	16	0.0037
B Analyses of plaques from clone 15			
15-1	0.00006	15-11	0.0001
15-2	0.00004	15-12	0.0004
15-3	0.0002	15-13	0.004
15-4	0.000006	15-14	0.002
15-5	0.000017	15-15	0.001
15-6	0.00007	15-16	0.003
15-7	0.00008	15-17	0.0002
15-8	0.007	15-18	0.001
15-9	0.0004	15-19	0.005
15-10	0.00012	15-20	0.0003
C Analyses of plaques from clone 10			
10-1	0.77	10-11	0.93
10-2	0.58	10-12	0.74
10-3	0.64	10-13	0.41
10-4	0.53	10-14	0.68
10-5	1.3	10-15	0.93
10-6	1.25	10-16	0.71
10-7	0.35	10-17	0.9
10-8	1.1	10-18	2.5
10-9	1.6	10-19	0.95
10-10	0.9	10-20	1.14

It is concluded that the majority of the clones arising on N agar from T4 (5/10/45) contain particles which can utilize L-tryptophan as a cofactor.

Previous work has shown that once a T4 particle requiring activation by a cofactor is adsorbed on B, the resulting virus-host complex is efficient in forming plaques on F agar at 37 C (Anderson, 1945). In order to determine whether

such plaques contain virus particles that require cofactor or not, a series of such plaques were analyzed. The virus particles in them were found to have low efficiencies of plaque formation on F agar.

The majority of the virus particles in the T4 (5/10/45) formed plaques of the fuzzy r+ type (Hershey, 1946). In order to obtain clear, more easily counted plaques for other work, a single clear plaque of the r type was isolated from an N agar plate on which T4 (5/10/45) had been assayed. It was added to a shaker flask containing actively growing B ( $\sim 10^8$  per ml) in 1,500 ml of F medium. The lysate assayed  $3.6 \times 10^{10}$  particles per ml and  $\sim 10^5$  particles per ml on N and F agar, respectively. Concentrated by differential centrifugation at 1,000 rpm and 10,000 rpm, the partially purified virus was filtered. Designated T4r (3/10/47), the resulting concentrate assayed  $3 \times 10^{11}$  particles per ml and  $4 \times 10^5$  particles per ml on N and F agar, respectively.

Individual plaques from this more homogeneous stock were also analyzed. A series of 8 F plates were poured with various total numbers (between  $2.5 \times 10^4$  and  $5 \times 10^9$ ) of virus particles together with a parallel series of 5 N plates, containing 40 to 400 particles from T4r (3/10/47). After incubation at 37 C for 18 hours, contact prints were made of each of the petri dishes to record the sizes of the plaques. A series of 40 plaques of varying sizes and morphologies were then picked from the F plates, and 10 plaques from the N plates. The suspended plaques were assayed on F agar and on N agar. As may be seen from figure 1, a fair correlation between the assays on N agar and the diameters of the plaques on F agar was obtained. On the other hand, no correlation between the numbers of virus particles in the plaques and the cofactor requirement of the predominating virus was noted. Nor did we detect consistent correlations between other aspects of the morphologies of these plaques (such as fuzziness or halos) with the cofactor requirements of the virus contained in the respective clones.

Nine of the 40 plaques taken from F agar gave assays on F agar at 37 C that were comparable to their assays on N agar. The remaining 31 plaques gave very low counts on F as compared to N agar. As may be seen from figure 1, no correlation between plaque size and the character of the virus was obtained.

Since a total of  $10^8$  virus particles had to be plated to produce about 80 plaques on F agar, the efficiency with which the cofactor-requiring fraction forms plaques on F agar may be estimated to be about

$$\frac{31}{40} \times \frac{80}{10^8} = 6 \times 10^{-7}$$

As was to have been expected, none of the plaques isolated from N agar plates gave comparable assays on F and N agar. Further analyses of this material are given in a following paper (Anderson, 1948b), in which the effect of temperature and nutrient on plaque formation by these clones was studied.

*Absorption of T4 stocks on B* Clearly the plaques isolated from F agar are of two types: some contain predominantly cofactor-requiring virus, others contain virus that appears not to require added cofactor. The question arises: Do all



the plaques that arise on F medium originate from cofactor-requiring types and owe their character to early mutations of the virus to nonrequirement followed, perhaps, by selection of these types? Or do the nonrequiring clones arise from

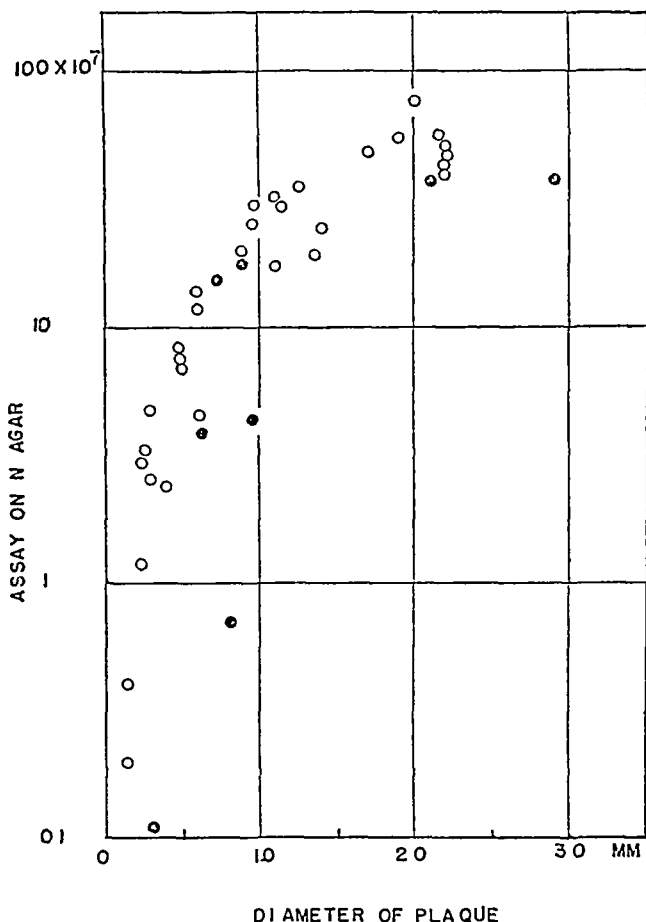


FIG 1 CORRELATION BETWEEN THE NUMBERS OF VIRUS PARTICLES IN F PLAQUES (ORDINATES) WITH THE MEASURED DIAMETERS OF THE PLAQUES (ABSCISSAE)

Two types of plaques are distinguished in the graph. O, plaques containing a predominance of particles that require cofactor, ●, plaques containing a predominance of particles that do not require added cofactor for the efficient production of plaques on F agar at 37 C. No correlation between cofactor requirement and plaque size was noted.

particles in the initial population that do not require added cofactor for efficient plaque production and inherit this character from them? If the latter situation exists it should be possible to remove the nonrequiring type from a population by adsorbing it on the host in the absence of added cofactor, and so reduce the assay of the stock virus on F agar. This was done as follows:

To 10 ml of a 5-hour, aerated, 37 C B culture in F medium ( $5 \times 10^9$  bacteria

per ml) was added 0.03 ml of T4 (5/10/45). After 10 minutes had been allowed for adsorption, the mixture was assayed on F and on N agar. The B+ adsorbed T4 was then removed by centrifugation and filtration. This treatment reduced the assay of the filtrate on F agar by 95 per cent, whereas that on N agar was unchanged. An analogous experiment in which the T4 suspension was absorbed on B three times in succession resulted in the removal of 98.7 per cent of the T4 that formed plaques on unfortified F agar. We conclude that virus of the non-requiring type pre-existed in T4 (5/10/45).

In three analogous experiments, T4 (5/10/45) was mixed with 24-hour B grown to saturation in aerated F culture at 37°C. With such B cultures adsorption is slow (Delbrück, 1940), and negligible fractions of the particles forming plaques on F agar were removed. However, the addition of 0.8 µg L-tryptophan per ml to these absorption mixtures enhanced the adsorption of these particles. In the three experiments, centrifugation of adsorption mixtures containing tryptophan removed 98, 93, and 93 per cent, respectively, of the T4 that was able to form plaques on F agar. The assays on N agar were again unchanged by absorption in the presence of these minimal amounts of tryptophan.

The results of these experiments indicate that the fraction of T4 that forms plaques on F agar at 37°C can be removed from a population of T4 virus particles. It has been further demonstrated that this fraction has an enhanced rate of adsorption on the host in the presence of L-tryptophan.

#### DISCUSSION

We have found that the virus in our stock cultures of T4 can be divided into at least two types—one producing plaques efficiently on minimal media at 37°C and the other doing so only in the presence of an added adsorption cofactor such as L-tryptophan. The response of the stock cultures to tryptophan would at least in part be determined by the proportions of the various types of virus which they contain.

Delbrück (1948) has added at least two types to the deficient forms of T4. T4,11 is like our tryptophan-deficient strains, but unlike ours its adsorption is strongly inhibited by traces of indole. The adsorption of another, T4,12, is also strongly inhibited by indole, and requires  $\text{Ca}^{++}$  in addition to a cofactor like tryptophan for adsorption. His T4,1 requires no added cofactor at 37°C, but the effect of temperature on plaque formation has not been studied (Anderson, 1948b).

The strains of T4 that are efficient in forming plaques on F agar probably arose by mutation of the cofactor-requiring type during its multiplication on B. To prove this we should have to show that the proportion of this fraction is highly variable in a series of cultures initiated by particles that are identical in their cofactor deficiency (Luria and Delbrück, 1943; Luria, 1945). The irregularities in the efficiencies of plating isolated deficient clones on F agar, as observed in table 1, may well be a reflection of this mutation effect. However, it did not seem worth while in the absence of more detailed knowledge of the mechanism of virus proliferation, plaque formation, and the factors influencing selection of

mutant strains to investigate the rate of mutation of T4 at this time (Hershey, 1946)

It may seem odd that the cofactor-requiring fractions should be able to form *any* plaques on B grown on unfortified F agar. However, it should be pointed out that the bacterial synthesis of tryptophan or other cofactors could well result in the presence of enough cofactor in the agar to activate small fractions of the deficient virus for adsorption and the initiation of plaque formation.

The fractions that are efficient in the production of plaques on F agar at 37 C probably require cofactor for activation too, but can utilize more effectively the low concentrations provided by the bacterial metabolism. The fact that L-tryptophan enhances their rate of adsorption on old bacteria lends support to this idea and at the same time provides a possible explanation for the slowness of virus adsorption on nonproliferating cells in exhausted media. Further indications of the true requirements of these fractions came from studies of the decreased efficiency with which they form plaques on F agar at low temperatures as described in the following paper (Anderson, 1948b). A survey of the effects of nutrients and temperature on the rates of adsorption of other viruses on bacteria that have passed the stationary stage of growth might well uncover unsuspected cofactor phenomena in their activities.

#### SUMMARY

A stock preparation of the bacteriophage T4 contained  $3.9 \times 10^{11}$  plaque-forming particles per ml on its host *Escherichia coli* strain B, grown on Difco nutrient agar (N), but only  $2.6 \times 10^8$  particles per ml forming plaques on B grown on ammonium lactate agar (F).

Individual plaques or clones from this stock appearing on N agar were suspended in F medium and assayed. They gave much lower counts on F agar than on N agar, and this characteristic persisted in subclones from these plaques, whether formed on F or on N agar.

The majority of individual clones appearing in assays of the stock on F agar, as well as their subclones, whether they arose on B on F or on N agar, produced as many plaques on F agar as they did on N agar.

The particles in the stock that are efficient in forming plaques on F agar can be removed by absorption on the host in F medium, but more effectively in the presence of the adsorption cofactor for T4, L-tryptophan.

We conclude that the degree of cofactor requirement is inherited in clones of T4.

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# THE INFLUENCE OF TEMPERATURE AND NUTRIENTS ON PLAQUE FORMATION BY BACTERIOPHAGES ACTIVE ON *ESCHERICHIA COLI* STRAIN B<sup>1</sup>

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Counting plaques is the most precise of the known procedures for assaying bacteriophage preparations. In this method, the preparation is diluted, and an aliquot containing a few hundred active virus particles is spread over the surface of hardened agar with  $10^7$  to  $10^9$  host bacteria. Normally, each active virus particle infects one of the bacteria on the growing smear, and when the infected cell lyses, the many new particles released carry the infection to other cells in the neighborhood. This process continues in an ever-widening chain reaction, until the stationary phase of bacterial growth is reached. At this point, infected cells no longer liberate virus. Whether or not the lytic reaction spreads to produce a visible hole or plaque in the bacterial smear thus depends on the outcome of a race between bacterial growth and the chain of lytic reactions involving the virus. Obviously, many factors can conceivably influence the outcome of the race: the rate of virus diffusion and adsorption on host cells, the ratio of the time of lysis of infected cells to the time of division of uninfected cells, and the number of virus particles liberated per cell are a few of the factors that might be involved. Previous studies have shown that to these factors must be added the activation of certain viruses by adsorption cofactors.

Some bacteriophages form plaques on bacterial smears under conditions in which they might not be expected to do so. For example, certain strains of the bacteriophages T4 and T6, active on strain B of *Escherichia coli*, are not adsorbed on their host unless activated by a cofactor such as L-tryptophan. Yet, once adsorbed, these viruses are able to produce plaques at 37°C on smears of B grown on agar containing ammonium lactate as the sole source of nitrogen and carbon. This seemed particularly odd since over 99 per cent of the virus particles isolated from such plaques still required added cofactor for the efficient formation of plaques on the minimal medium (Anderson, 1948b). One might suppose that these particles acquired cofactor from the bacterium—either from the lysed host cell or from the growing bacteria in the smear on which the plaque is formed.

After it was found that the activation of T4 by L-tryptophan was strongly dependent on temperature (Anderson, 1948a), we undertook a brief study of the temperature dependence of plaque formation by T4 and T6 on minimal agar. After striking dependences were found, we extended the investigation to other

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viruses of the T set (Demerec and Fano, 1945) in a search for other cofactor phenomena. The results of these studies are presented here.

#### MATERIALS

Strain B of *E. coli*, the minimal ammonium lactate media (F), and the "complete" nutrient media (N) have been described previously (Anderson, 1945). The original stocks of T1 and T2 were kindly supplied by Luria and Delbrück. The parent stocks of T3, T4, T5, T6, and T7 were kindly furnished by Demerec and Fano (1945). All dilutions and platings of viruses for assay were made in

TABLE 1

*The number of plaques formed by the bacteriophage T4 on its host (E. coli strain B) grown on synthetic (F) agar and nutrient (N) agar at various temperatures*

DILUTION OF STOCK VIRUS	TYPE OF AGAR	TEMPERATURE OF INCUBATION						
		7 C	16 C	25 C	31 C	31 C	37 C	37 C
1 10,000	F	No	470*	800	Confluent lysis			
1 200,000	F	bacterial	33	79	163	142	263	273
1 40,000,000	N	growth	595	625	615	650	650	623

\* Each number represents the average count for two plates.

TABLE 2

*Effect of temperature on the formation of plaques by stock preparations of T4 and T6*

STOCK	TEMPERATURE	ASSAY ON F AGAR	ASSAY ON N AGAR
T4(10/20/45)	14 C	Less than $10^3$	$1.3 \times 10^{12}$
	37 C	$2.2 \times 10^{10}$	$1.1 \times 10^{12}$
T4(1/9/45)	14 C	$1.4 \times 10^{11}$	$1.9 \times 10^{11}$
	37 C	$2.6 \times 10^{11}$	$1.9 \times 10^{11}$
T6(1/10/45)	15 C	$4 \times 10^{10}$	$1.6 \times 10^{12}$
	37 C	$1.1 \times 10^{12}$	$1.1 \times 10^{12}$

liquid F medium in an air-conditioned room at 15 C. Pourings were made by the soft agar technique, originally described by Gratia (1936, cf. also Delbrück, 1946). The surface layers were of 0.7 per cent agar for F plates and 0.7 per cent N agar for N plates. Plates were poured in duplicate, two or more pairs of plates being incubated at each of the temperatures studied.

#### EXPERIMENTAL PROCEDURE

*T4 and T6* A stock preparation, T4 (5/10/45), which contained over  $10^{11}$  particles per ml able to form plaques on N agar was diluted 1 10,000, 1 200,000, and 1 40,000,000 in F medium. Fourteen F plates were poured with 0.1 ml from each of the first two dilutions and 14 N plates from the third. The plates were incubated at various temperatures in sets of six, two from each dilution of

T4 The numbers of plaques were counted each day for 5 days, although after 3 days the counts did not increase appreciably. It is well to note at this point that most of the plaques appearing on F agar from this stock at 37 C are produced by strains of T4 in the population that are efficient in forming plaques on F-agar at 37 C without the addition of a cofactor (Anderson, 1948b). Their efficiency in forming plaques on F agar decreases as the temperature is lowered, as is seen in table 1, but the efficiency with which the main cofactor-requiring fraction forms plaques on N agar is unchanged by temperature.

Two other stocks of T4 and one of T6 were assayed on F and on N agar at 14 C and 37 C. The results presented in table 2 show that one stock, T4 (10/20/45), gave markedly lower assays on F at 14 C than at 37 C, whereas the assay on N agar remained unchanged. The other stock gave essentially similar assays on F and on N at both temperatures. The stock of T6 virus that gave equivalent assays at 37 C dropped in apparent titer at 15 C on F agar, but not on N agar.

TABLE 2

*Effect of temperature on plaque formation by adsorption mixtures of E. coli strain B and T4 activated by L-tryptophan*

ACTIVATING CONCENTRATIONS OF L-TRYPTOPHAN $\mu\text{G}/\text{MLF}$	NUMBER OF PLAQUES FORMED PER ML OF ADSORPTION MIXTURE	
	On F plates at 15 C	On F plates at 37 C
0.1	—	$5 \times 10^1$
0.2	$5 \times 10^1$	$3.3 \times 10^2$
0.5	$2 \times 10^4$	$1 \times 10^5$
2.0	$2.8 \times 10^6$	$1.4 \times 10^7$

In addition, a series of 50 clones, nos. 101 to 150, were isolated from plates on which T4r (3/10/47) had been assayed (Anderson, 1948b). None of them gave significantly lower assays on N plates incubated at 14 C than on N plates incubated at 37 C. Of the nine clones from F plates that gave comparable assays on F and N agar at 37 C only one (no. 127) continued to do so at 14 C and then with a markedly lower plaque count.

Since we have been employing the assay on F agar as a measure of the concentration of infected B in adsorption mixtures of B and T4 activated by L-tryptophan, it seemed desirable to determine the temperature coefficient of plaque formation by such infective centers. Accordingly, adsorption mixtures analogous to those used previously were prepared (cf. figure 4 of Anderson, 1948a) in which the virus was activated at 37 C by different tryptophan concentrations, but adsorbed on B under the same low tryptophan concentrations of 0.02  $\mu\text{g}$  per ml. After 5 minutes had been allowed for adsorption, during which time all the unadsorbed virus had lost its activation, the mixtures were diluted in F medium and assayed on F and N plates. After the agar had hardened, one set of plates was held at 15 C and another set incubated at 37 C. The assays on N plates ( $4 \times 10^7$  per ml) were again unaffected by the temperature of incubation,

but the counts on F plates at 15 C were only about 20 per cent of those at 37 C (table 3) We see that the chain of reactions following the adsorption of the primary virus particle on its host is blocked at low temperatures on the minimal medium

The fact that the plating efficiency for the stocks, clones, and infected cells remained constant on N agar but decreased markedly at lower temperatures on F agar suggested that a nutritional deficiency arises on the minimal medium as the temperature is lower To test the possibility that the clones required an

TABLE 4

*Numbers of plaques produced by clones of T<sub>4</sub>r on (F) agar, on F agar, + 20 µg L tryptophan per ml (T), and on complete (N) agar at 37 C and at 14 C*

CLONE	FRACTION OF CLONE PLATED*	PLATES INCUBATED AT 37 C			PLATES INCUBATED AT 14 C		
		F	T	N	F	T	N
105	1/800,000	36	138	167	0	83	115
108	"	0	146	245	0	237	223
109	"	208	363	199	0	99	201
111	"	580	531	497	0	373	381
116	"	0	200	185	0	157	151
120	"	426	441	486	0	295	373
122	1/4,000	213	288	290	0	157	198
125	1/400	308	495	339	0	335	488
127	1/80,000	106	83	62	8	35	68
128	"	364	139	300	0	231	272
130	1/40,000	490	600	500	(0)	377	110

\* In 0.05 ml

TABLE 5

*Ratios of assays on F agar to assays on N agar at 15 C and 37 C for viruses active on E. coli*

TEMPERATURE OF INCUBATION	VIRUS				
	T1	T2	T3	T5	T7
15 C	0.05	0.59	0.82	0.86	0.004
37 C	0.2	1.5	1.3	0.88	0.54

added adsorption cofactor at 14 C, a parallel set of assays of a group of clones was carried out on F agar, on N agar, and on T agar (F agar containing 20 µg L-tryptophan per ml) The results are presented in table 4 Again the assays on F agar dropped to low values at 14 C The assays on T agar were comparable to those on N agar, indicating that L-tryptophan in the agar made up the nutritional deficiencies Two clones, 108 and 116, both cofactor-deficient at 37 C, were included in the set of nondeficient clones They too gave equivalent numbers of plaques on T agar and on N agar at 14 C

*T1, T2, T3, T5, and T7* Our stock preparations of the remaining viruses in the set under study were assayed on F agar and on N agar at 37 C and at 15 C



The results are given in table 5. Again no variations in the assays on N agar were noted, whereas marked temperature dependences are displayed in the assays of T1 and T7 on F agar. Again, nutritional deficiencies are suggested by these results. Preliminary tests indicate that isoleucine, methionine, and norleucine added separately to F agar promote plaque formation by T1. Isoleucine, leucine, methionine, and norleucine increase the efficiency of plating T7 on F agar at 15 C.

#### DISCUSSION

It would appear from the following results that the formation of plaques by T4 on minimal F agar at low temperatures is limited by the lack of adsorption cofactors for these viruses. (1) L-Tryptophan, which is an adsorption cofactor for T4, raises the assays to those obtained on complete N agar. (2) The rate of adsorption of those strains that are efficient in producing plaques on F agar at 37 C is enhanced by L-tryptophan (Anderson, 1948b). (3) In the range available for study the temperature dependence of activation of T4 by L-tryptophan (Anderson, 1948a) parallels the temperature dependence of plaque formation observed here.

The strains of T4 that are efficient in forming plaques on F agar at 37 C but inefficient at 14 C are particularly interesting, for their behavior suggests that they derive sufficient cofactor from the medium containing metabolizing host cells at 37 C to be activated. At 14 C, however, the strains studied are considered to have little chance to form plaques because at these temperatures the cofactor concentrations provided by the cells are insufficient for the initial activation of the virus.

Putting these views in other terms, we may state that on F agar at 14 C the bacteria are resistant to these strains, at 37 C they are sensitive. At 37 C they are sensitive because they make and liberate enough cofactor to activate the virus, at 14 C these quantities of cofactor are insufficient to activate an appreciable fraction. The concentrations of cofactor involved may be estimated from the curves of activation of T4 as a function of the tryptophan concentration (Anderson, 1948a) to be equivalent to 0.1  $\mu$ g L-tryptophan per ml or less. Whether L-tryptophan or some other cofactor is responsible for T4 activation on F agar we do not yet know. However, from the steepness of the activation curves it may be seen that the liberation of a little more cofactor by the bacteria would have made them sensitive to a much larger proportion of the virus in our stocks at 37 C. Here we have a single facet in a physiological basis for sensitivity and (if the bacteria were to liberate less cofactor) resistance of the host cells to viruses.

Another basis for resistance that is somewhat complementary to that discussed above has been uncovered by Delbruck (1948). Certain strains of T4 that he isolated, like ours, require adsorption cofactors, but the adsorption or activation of these strains is blocked by indole in minute quantities. (The activation and adsorption of our strains were only slightly inhibited by large concentrations of indole.) Now *E. coli*, in metabolizing tryptophan, liberates indole, so that under

certain conditions Delbruck observes the metabolic processes of the host creating resistance to the virus

Some strains of T4 have an efficiency of plaque formation at 37 C that is only  $10^{-6}$  or so on F agar (Anderson, 1948b). Yet a virus-host complex formed by one of these strains has an efficiency of plaque formation of nearly unity at 37 C (Delbruck, 1948). How are the virus particles liberated from these cells activated to carry the infection to other cells in the neighborhood? Only 30 to 40 virus particles are liberated from B on F medium. One might estimate their chances of continuing the infection to one of the surrounding cells to be only 30 to 40 times  $10^{-6}$ . The chances that a chain of such highly improbable reactions would proceed to the destruction of enough bacteria to make a visible plaque would be infinitesimal indeed. We can only surmise that on lysis the complex liberates cofactor sufficient in activity to permit an appreciable fraction of the liberated virus to infect neighboring cells.

That this process is amazingly efficient may be appreciated from the following additional observations: (1) The infrequent plaques formed by such deficient clones on F medium are indistinguishable from plaques formed by clones that are not deficient at 37 C, both in morphology and in the amount of virus contained (Anderson, 1948b). (2) The efficiency of plaque formation by the non-deficient particles at 37 C is reduced by a factor of 100 or more, whereas efficiency of plaque formation by cells infected with deficient strains is reduced by a factor of only 5 on lowering the temperature from 37 C to 15 C.

The viruses discussed so far are rather "organismal" in morphology. T4 and T6, tadpole-shaped with membranes surrounding internal structures in the heads (Anderson, 1946), might be expected to display nutritional requirements like independently multiplying organisms. Here we have observed cofactorlike phenomena in other unrelated and structurally more primitive viruses like T7 (a 400 A sphere—Delbruck, 1946) and T1 (a 500 A sphere with an attached thin tail—Luria and Anderson, 1942; Luria, Delbruck, and Anderson, 1943). One suspects that the phenomena might be prevalent among viruses, but screened from observation by the synthesis of specific cofactors by sensitive cells in adequate quantities for virus activation.

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#### SUMMARY

At 15 C *Escherichia coli* strain B, grown on synthetic medium, becomes resistant to some of the viruses that are active on it at 37 C.

Stocks and clones of the bacteriophage T4 were assayed on agar containing ammonium lactate as the sole source of carbon and nitrogen (F) and on Difco nutrient agar (N). Parallel sets of plates were incubated at 15 C and at 37 C.

On N agar no effects of the temperature of incubation were noted, but on F

agar all stocks but one showed efficiencies of plaque formation at 14 C that were only 1/100th of those at 37 C. All clones of the virus tested displayed this phenomenon, including those that were efficient in forming plaques on F agar at 37 C.

In all cases tested, the efficiency of plaque formation by T4 on F agar at 15 C was brought to unity by the addition to F agar of 20  $\mu$ g per ml of L-tryptophan, an adsorption cofactor for T4.

A comparison of these and previous results suggests that the metabolism of the bacteria in minimal medium furnishes sufficient cofactor for the activation and adsorption of those strains of T4 that are efficient in producing plaques on F at 37 C, but that these amounts of cofactor in many cases are not sufficient at 15 C.

A survey of the other viruses in the T set disclosed similar inefficiencies at 15 C in the formation of plaques by our stocks of T1 and T7. These deficiencies were at least partially overcome by the addition of certain amino acids to the F agar.

The possibility that the resistance of host cells to viruses is at least in part determined by their metabolism is discussed in the light of these results.

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# CULTURAL CHARACTERISTICS OF DONOVANIA GRANULOMATIS

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In a previous paper by Dunham and Rake (1948) the successful propagation of *Donovania granulomatis* on artificial media, after adaptation, was described. Since that publication the strain has been maintained for additional passages on the media containing equal parts of 3 per cent agar in beef heart infusion broth and modified Levinthal's stock broth. The present communication will describe the later behavior of this strain together with certain details of gross and microscopic morphology.

## MATERIALS AND METHODS

The following artificial media have been employed

(1) *Beef heart infusion broth*

- (a) Trim fat from fresh beef hearts and grind the lean meat
  - (b) Add 1,000 ml of distilled water per 1 pound of heart
  - (c) Place in icebox overnight and the next morning heat to 85 C for 30 minutes
  - (d) Filter through Prat-Dumas filter paper
  - (e) Add 1 per cent Pfanstiehl peptone, 0.5 per cent NaCl, and 0.03 per cent glucose
  - (f) Boil for 30 minutes
  - (g) Adjust pH to 7.6 with NaOH
  - (h) Filter through Whatman no. 2 filter paper
  - (i) Sterilize at 15 pounds for 20 minutes
- (Used as broth, or as agar with 1.5 per cent Difco agar)

(2) *Rabbit blood agar or broth*

Add 4 per cent fresh citrated rabbit blood to the foregoing beef heart infusion media. In the case of the agar, it is cooled to 40 C before the blood is added and the plates are poured immediately thereafter.

(3) *Levinthal beef heart medium*

- (a) To 425 ml of medium (1) (broth) described above add 75 ml of defibrinated sheep or rabbit blood
- (b) Mix and bring to boil for 5 minutes until brown clot forms
- (c) Filter first through coarse filter paper and then through Seitz E K pads to sterilize

To prepare agar from this broth mix equal parts of

- (a) Medium (1) (broth) described above containing 3 per cent Difco agar, and
- (b) the Levinthal broth

All the work with eggs has been done in embryonated chicken's eggs, of 6-day incubation, inoculated by the yolk sac route.

For the electron microscope studies an RCA microscope type EMU has been used. For shadow casting and replica techniques the suggestions of Williams and Wyckoff (1946) and Hullier and Baker (1946) have been followed.

### RESULTS

The culture on Levinthal beef heart infusion agar has been maintained for 43 passages. For transfer, 1 ml of beef heart infusion broth is added to the tube, the colonies are rubbed off into the broth, and 0.1 ml is transferred to the new slant. Cultivation in the later passages occurred more readily than it did originally, but it has never been luxuriant. Colonies appeared within 48 hours scattered all over the slant. Colonies were at first translucent and shiny. They increased in size until the larger measured some 1.5 mm in diameter, gradually becoming gray and, later, brownish in color. In the later passages the stringy mucoid character noted earlier was, to a large degree, lost.

Throughout the whole series of passages on this agar medium, on every occasion that a transfer was made to a new slant, i.e., every 7 to 12 days, a tube of beef heart infusion broth was also inoculated with 0.05 ml of the culture suspension. For the first 25 passages no growth occurred in these broth tubes in the 12-day period over which they were held. On the twenty-sixth passage growth appeared in 1 tube of broth of the 3 used for the 3 separate subcultures. The growth did not appear before the sixth day, it was scanty and appeared first in the surface layers of broth. By the tenth day the organisms were lysing and the turbidity in the tube was clearing. Since the twenty-sixth passage similar transfers to the beef heart broth tubes have usually, but not invariably, produced evidence of growth similar to that just described. With such a broth culture originating from the material transferred from the twenty-seventh passage, serial transfers in beef heart broth were originated and have now been carried for 38 passages. During these passages the growth characteristics have remained unchanged. Growth was scanty. It appeared in about 48 hours and increased for 5 to 7 days with some precipitation of larger clumps to the bottom of the tube, after which the turbidity tended to clear. Transfers have been made at from 4- to 13-day intervals.

Subculture of this beef heart broth culture was made after 14 and 38 serial transfers in this medium to Levinthal's agar, rabbit blood agar, and beef heart agar. All subcultures from the fourteenth transfer grew out more readily on the Levinthal's agar (first growth apparent in 6 days) than on the rabbit blood agar (first growth apparent in 11 days) or on the beef heart agar (first growth apparent in 17 days). Subculture from the thirty-eighth transfer grew out on Levinthal's agar in 4 days, on rabbit's blood agar in 7 days, and on beef heart agar in 9 days.

The microscopic appearance of the organism in beef heart broth, whether under the light microscope or the electron microscope (see below), did not differ from *D. granulomatis* passed in the yolk of the embryonated chicken's egg or on Levinthal's agar. Under the light microscope the organisms of the beef heart broth

culture showed themselves to be pleomorphic gram-negative bacilli with characteristically prominent polar granules. Many very long chains, looking like coiled filamentous forms, were present, and single organisms tended to be comma-shaped. In older organisms one could often see clearly the retraction of the bacterial substance from the cell wall.

Both the culture on the Levinthal agar slants (after 25 passages) and the culture in the beef heart (sterility) broth derived from the fortieth passage on Levinthal agar slants have been reinoculated into 6-day embryonated chicken's eggs by the yolk sac route, and maintained there by passage at 3- or 4-day intervals. The

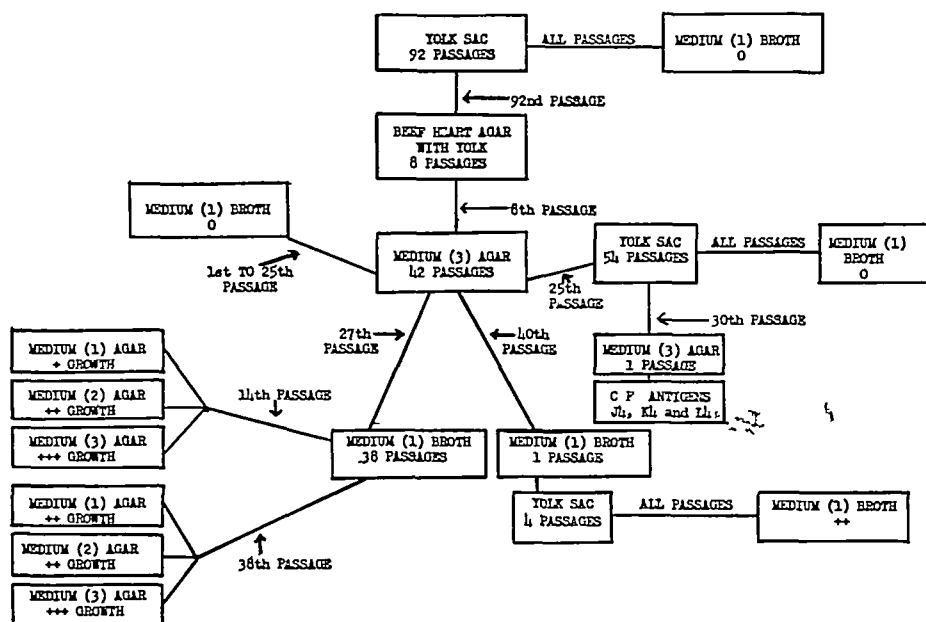


FIG 1 SCHEMA OF PASSAGES WITH *D. GRANULOMATIS*

Media (1), (2), and (3) as given under Materials and Methods

+ = Slight growth delayed about 2½ weeks

++ = Slight growth appearing more promptly, in about 1½ weeks

+++ = Moderate growth appearing in about 2 to 3 days

behavior of the first of these egg strains has differed in no way from that of the original culture of *D. granulomatis*, received from Dr. Anderson, when that was carried in eggs by us, whereas the second has differed only in respect to the behavior of small inocula, of the yolk used for egg-to-egg passage, into beef heart broth. Scant growth, similar to that in the broth culture initiating this egg strain, occurred with all passage material tested. Such growth has never occurred from other egg passage material. Samples of this passage yolk also gave good growth on Levinthal's agar slants in 2 days, and slight growth on rabbit blood agar slants or beef heart infusion slants in 6 or 7 days.

After 30 egg passages the egg strain initiated from the twenty-fifth Levinthal agar passage (see above) was replanted on Levinthal agar slants, and these were

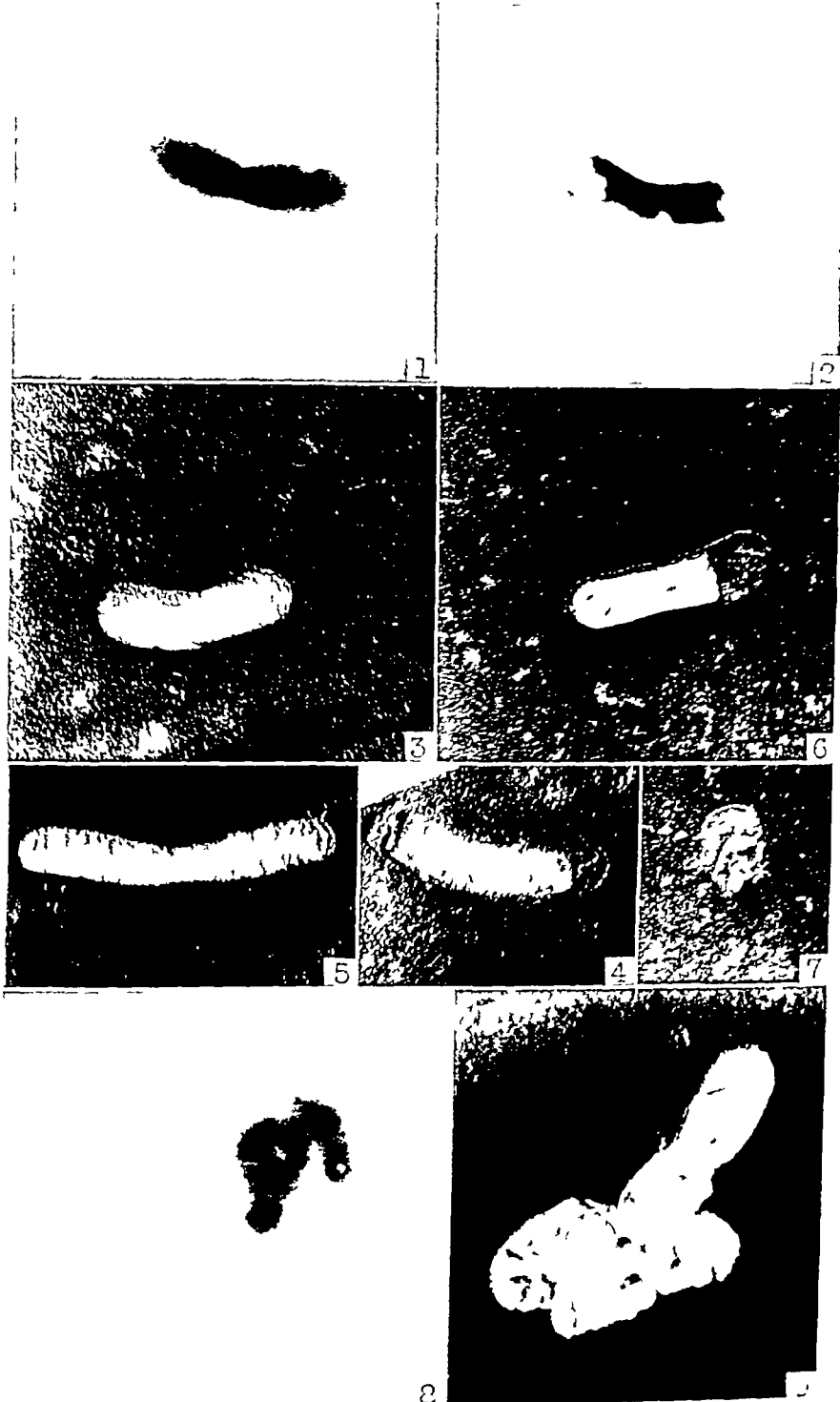


FIGURE 2



used for preparing an antigen for complement fixation tests with known granuloma-positive and known granuloma-negative antisera. The antigen behaved in every respect like other antigens described elsewhere (Dunham and Rake, 1948, Rake, 1948) prepared from the original egg (yolk sac) strain that had never been on artificial media or from the Levinthal slant passage strain.

The somewhat complicated history of the various materials discussed above is shown diagrammatically in figure 1.

#### ELECTRON MICROSCOPY

*D. granulomatis* organisms growing under the different conditions described above have been examined repeatedly under the electron microscope, utilizing the techniques mentioned under Materials and Methods. In general, the same appearance has been found with organisms produced by the various cultural methods, and illustrations are given of organisms from yolk sac cultures, from the colonies on yolk beef heart infusion agar (Dunham and Rake, 1948) and Levinthal's agar, and from the scanty growth in beef heart infusion broth.

In figure 2, nos 1 to 7 are of organisms from yolk sac cultures, in figures 2-3, nos 8 to 12 are from cultures on yolk beef heart infusion agar (Dunham and Rake, 1948), in figure 3, nos 13 to 16 are from cultures on Levinthal beef heart infusion agar, and in figures 3-4, nos 17 to 25 are from cultures in beef heart infusion broth. In figure 4, nos 26 to 29 are of *Klebsiella pneumoniae* growing in tryptone broth. All magnifications are the same ( $\times 8,850$ ) except for figure 3, nos 13 ( $\times 8,620$ ), 14, and 15 ( $\times 7,250$ ), which unfortunately were taken at primary magnifications such that, for technical reasons, they could not be brought into exact agreement with the other micrographs.

In general, there is agreement in morphology between organisms from different media. No definite encapsulation is to be seen. The capsulelike appearance in figure 2, nos 1 and 5, for example, would seem to be due to slight separation of the plasma membrane from the cell wall, which is a very characteristic and pro-

#### FIGURE 2

No 1 *D. granulomatis* From yolk sac culture. The plasma membrane has retracted from the cell wall. One polar granule and other bodies.  $\times 8,850$ .

No 2 *D. granulomatis* From yolk sac culture. Comma shaped bacillus. Retraction of plasma membrane.  $\times 8,850$ .

No 3 *D. granulomatis* From yolk sac culture. Comma shaped bacillus. Gold shadowed 22.5 mg,  $11^{\circ}32'$  angle, 10 cm distance.  $\times 8,850$ .

No 4 *D. granulomatis* From yolk sac culture. Comma shaped bacillus. Retraction of plasma membrane. Polar granules. Gold shadowed 22.5 mg,  $11^{\circ}32'$  angle, 10 cm distance.  $\times 8,850$ .

No 5 *D. granulomatis* From yolk sac culture. Wrinkled cell surface. Gold shadowed 22.5 mg,  $11^{\circ}32'$  angle, 10 cm distance.  $\times 8,850$ .

No 6 *D. granulomatis* From yolk sac culture. Retraction of plasma membrane. Polar granule. Wrinkled cell surface. Gold shadowed 22.5 mg,  $11^{\circ}32'$  angle, 10 cm distance.  $\times 8,850$ .

No 7 *D. granulomatis* From yolk sac culture. Autolysed cell showing cell wall and polar granules. Gold shadowed 22.5 mg,  $11^{\circ}32'$  angle, 10 cm distance.  $\times 8,850$ .

No 8 *D. granulomatis* From yolk beef heart agar. Polar granules and other bodies.  $\times 8,850$ .

No 9 *D. granulomatis* From yolk beef heart agar. Polar granules. Wrinkled cell surface. Gold shadowed 23.9 mg,  $10^{\circ}59'$  angle, 10.5 cm distance.  $\times 8,850$ .

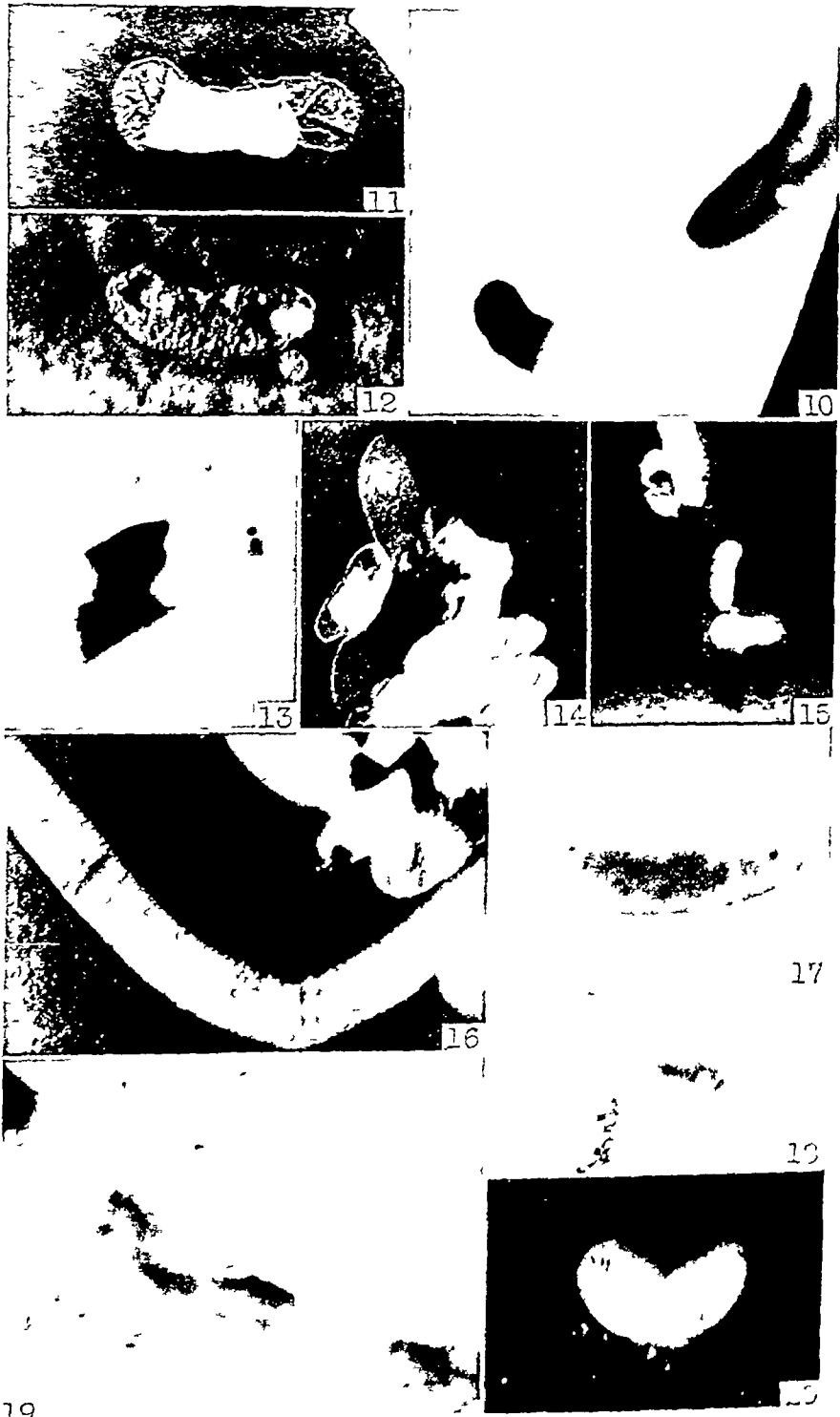


FIGURE 3

nounced feature in older organisms (figures 2-3, nos 2, 4, 6, 10, 11, 13, 14, and 19). As has been noted elsewhere (Dunham and Rake, 1948), the loss of capsular material occurred gradually during the original egg yolk passages and first transfers on artificial media, and has been associated with a loss of mucoid characteristics in yolk and colony. There is a definite indication of a "sticky" surface to the cell wall, however, and adherent particles or other material can be seen in figures 3-4, nos 15, 17, 18, 21, and 22. The cell wall, whether in the intact cell or in the autolysed specimens, often shows pronounced and characteristic wrinkling (figures 2-4, nos 5, 9, 10, 16, 21, 23, and 24).

Polar granules are prominent in the cytoplasm (figures 2-4, nos 1, 4, 6, 8, 9, 19, and 24), and they appear to be more resistant to lysis than the remainder of the cytoplasm, so that the cell wall and polar granules are often all or almost all that remain (figures 2-4, nos 7, 8, 12, 19, 24, and 25). Other bodies are seen not infrequently within the cytoplasm (figures 2-4, nos 1, 8, 17, and 22).

The organism is characteristically a short plump bacillus, but considerable pleomorphism is found. The length varies from  $1.5 \mu$  to  $4.5 \mu$  and the breadth from  $0.8 \mu$  to  $1.4 \mu$ . The significance of the still smaller bodies, seen for example in figure 3, nos 15 and 18, is uncertain. They measure  $1.4 \mu$  to  $1.5 \mu$  in length and  $0.5 \mu$  to  $0.7 \mu$  in breadth and show all the characteristics of the larger forms. They are of such a size, of course, as would pass through a Berkefeld V or an even smaller filter. Their relationship to filterable organisms of possible etiological importance in granuloma inguinale (DeMonbreun and Goodpasture, 1933) has been discussed elsewhere (Rake, 1948).

Prior to division the elongated bacilli tend to become comma shaped (figure 3, nos 2, 3, 10, 11, 12, 17, and 20). In many cases the division is not complete, and chains of 15 or more bacilli occur stretching across many fields of the electron

FIGURE 3

No 10 <i>D. granulomatis</i>	From yolk beef heart agar	Retraction of plasma membrane
Wrinkled cell wall	$\times 8,850$	
No 11 <i>D. granulomatis</i>	From yolk beef heart agar	Retraction of plasma membrane
Wrinkled cell wall	Gold shadowed 23.9 mg, $9^{\circ}36'$ angle, 12 cm distance	$\times 8,850$
No 12 <i>D. granulomatis</i>	From yolk beef heart agar	Autolysed cell showing cell wall and polar granules
	Gold shadowed replica 23.9 mg, $12^{\circ}23'$ angle, 11 cm distance	$\times 8,850$
No 13 <i>D. granulomatis</i>	From Levinthal beef heart agar	Retraction of plasma membrane
	$\times 8,620$	
No 14 <i>D. granulomatis</i>	From Levinthal beef heart agar	Retraction of plasma membrane
Wrinkled cell surface	Small forms	Gold shadowed 21.2 mg, $11^{\circ}32'$ angle, 10 cm distance
	$\times 7,250$	
No 15 <i>D. granulomatis</i>	From Levinthal beef heart agar	Small forms
Accumulation of particles about cell surface	Gold shadowed 21.2 mg, $11^{\circ}32'$ angle, 10 cm distance	$\times 8,850$
No 16 <i>D. granulomatis</i>	From Levinthal beef heart agar	Long chain of bacilli
Wrinkled surface	Polar granules	Gold shadowed 21.2 mg, $11^{\circ}32'$ angle, 10 cm distance
	$\times 8,850$	
No 17 <i>D. granulomatis</i>	From beef heart broth	Comma shaped
bodies	$\times 8,850$	Small opaque
No 18 <i>D. granulomatis</i>	From beef heart broth	Small form
Accumulation of particles about cell surface	$\times 8,850$	
No 19 <i>D. granulomatis</i>	From beef heart broth	Accumulation of particles about cell surface
	$\times 8,850$	
No 20 <i>D. granulomatis</i>	From beef heart broth	Wrinkled cell surface
	Gold shadowed 22.9 mg, $10^{\circ}1'$ angle, 11.5 cm distance	$\times 8,850$

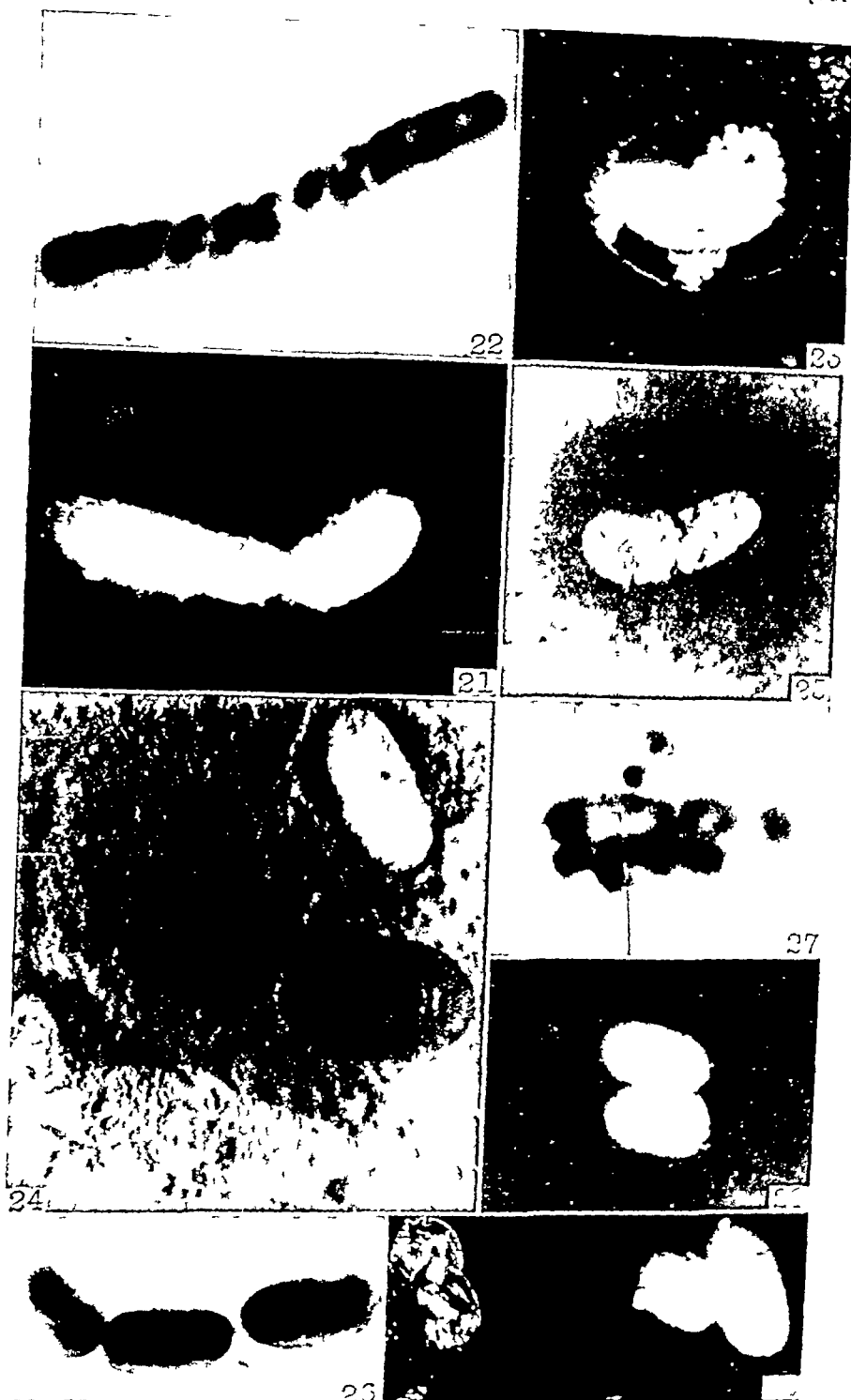


FIGURE 4

microscope (figure 3, no 16) These are, of course, the long coiled filamentous forms seen so frequently with the light microscope

For purposes of comparison, 4 micrographs of *K pneumoniae* (Friedlander's bacillus) are included (figure 4, nos 26 to 29) The general similarity between the two microorganisms is apparent It must be emphasized, however, that there is little cultural resemblance between the luxuriant growth of *K pneumoniae* and the, at present, scant growth of *D granulomatis* in the media tested

#### SUMMARY

*Donovania granulomatis*, after adaptation, grows on many artificial media Neither by antigenic composition (as tested with the complement fixation test) nor by morphological appearance under light or electron microscopes can these organisms growing on artificial media be distinguished from the original culture after repeated propagation in the yolk sac of the chicken embryo In our hands, however, luxuriant growth has never occurred on any artificial medium

As has been shown elsewhere (Rake, 1948) *D granulomatis* has antigenic relationships to *Klebsiella pneumoniae* Electron micrographs indicate a morphological similarity between the two organisms

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#### FIGURE 4

No 21	<i>D granulomatis</i>	From beef heart broth	Wrinkled surface	Gold-shadowed
22.9 mg,	10°1' angle,	11.5 cm distance	× 8,850	
No 22	<i>D granulomatis</i>	From beef heart broth	Accumulation of particles about cell surface	
		Internal bodies	× 8,850	
No 23	<i>D granulomatis</i>	From beef heart broth	Wrinkled surface	Gold shadowed
22.9 mg,	10°1' angle,	11.5 cm distance	× 8,850	
No 24	<i>D granulomatis</i>	From beef heart broth	Polar granules	Gold-shadowed
replica 23.9 mg,	12°23' angle,	14.0 cm distance	× 8,850	
No 25	<i>D granulomatis</i>	From beef heart broth	Polar granules	Gold-shadowed
22.9 mg,	10°1' angle,	11.5 cm distance	× 8,850	
No 26	<i>K pneumoniae</i>	From tryptone broth	Polar granules	× 8,850
No 27	<i>K pneumoniae</i>	From tryptone broth	Polar granules	Cell wall × 8,850
No 28	<i>K pneumoniae</i>	From tryptone broth	Gold shadowed 20 mg,	11°32' angle,
10 cm distance	× 8,850			
No 29	<i>K pneumoniae</i>	From tryptone broth	Polar granules in normal and autolysed cells	Gold shadowed 20.9 mg,
				11°32' angle, 10 cm distance × 8,850



# THE EFFECT OF SODIUM FLUORIDE ON THE METABOLISM OF CERTAIN MYCOBACTERIA

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Little is known about the effect of fluorides on bacteria. In large concentrations inorganic and organic fluoride compounds inhibit growth. Fluorine is not known to be an essential trace element for bacteria, fungi, or algae. Recently Davis and Dubos (1947) have shown that fluoride inhibits the action of a lipase on "tween 80" in concentrations that do not affect the growth of *Mycobacterium tuberculosis* H37RV. The following is an account of the effects of the fluoride ion on the metabolism of certain mycobacteria.

## EXPERIMENTAL RESULTS

Most of the experiments were done with a rapidly growing BCG strain of *M. tuberculosis* (ATC 8240). Similar effects were, however, obtained with *M. tuberculosis* (ATC 607) and the strain H37RV<sup>1</sup>. The BCG organisms were grown in Long's medium for 2 to 4 days. The masses were broken up and washed in water by centrifugation in a Hopkins tube. Even suspensions were made in M/20 Na-K-phosphate buffer at various hydrogen ion concentrations. Three to four mg dry weight of bacteria were used in each Warburg vessel, which contained a total volume of 2.0 ml. The oxygen uptake was measured in air at 37°C. Various samples of analytical grade sodium fluoride were used.

Figure 1 shows the effect of several concentrations of sodium fluoride on the oxygen uptake of washed suspensions of BCG at different hydrogen ion concentrations. A large increase in the rate of oxygen uptake occurs. For low concentrations of fluoride the increase is maximal at pH 6.0. At pH 6.7 a higher concentration of fluoride is necessary to produce a corresponding increase, and at pH 7.8 even high concentrations have only small effects. At pH 6.0 and 6.7, if the concentration of fluoride is too large, acceleration gives way to inhibition, but at pH 7.8 no inhibition occurs with the concentrations used. The fluoride ion is, therefore, more effective on the acid side of neutrality. The absolute increase in oxygen uptake for any concentration of fluoride depends on the number of bacteria present. Thus at pH 6.0 a concentration of fluoride that causes an increased O<sub>2</sub> uptake with 0.5 ml of a bacterial suspension may cause an inhibition when only 0.2 ml of suspension are used.

Equimolar solutions of sodium iodide, bromide, and chloride are without effect on the oxygen uptake. Under conditions that produce maximal acceleration with fluoride, equimolar concentrations of oxalate are without effect. This in-

<sup>1</sup> Obtained originally from the culture collection of the National Tuberculosis Association, Trudeau, New York.

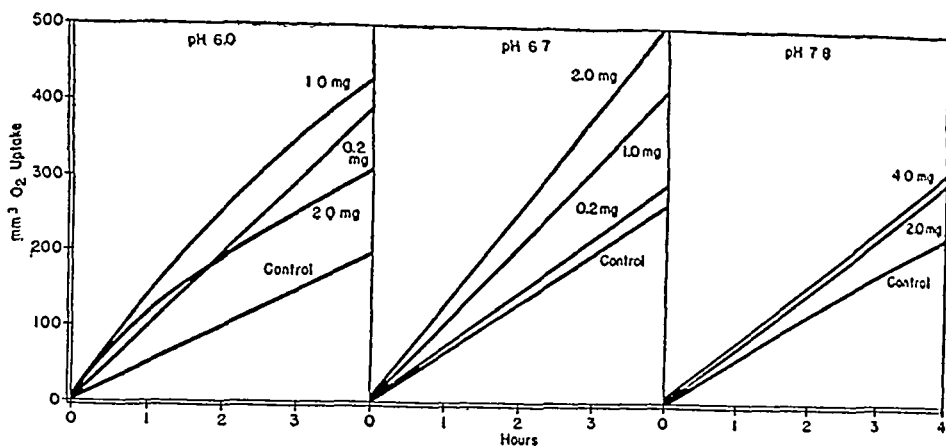


FIG 1 THE EFFECT OF DIFFERENT AMOUNTS OF NaF ON THE OXYGEN UPTAKE OF SUSPENSIONS OF MYCOBACTERIUM BCG AT 3 HYDROGEN ION CONCENTRATIONS

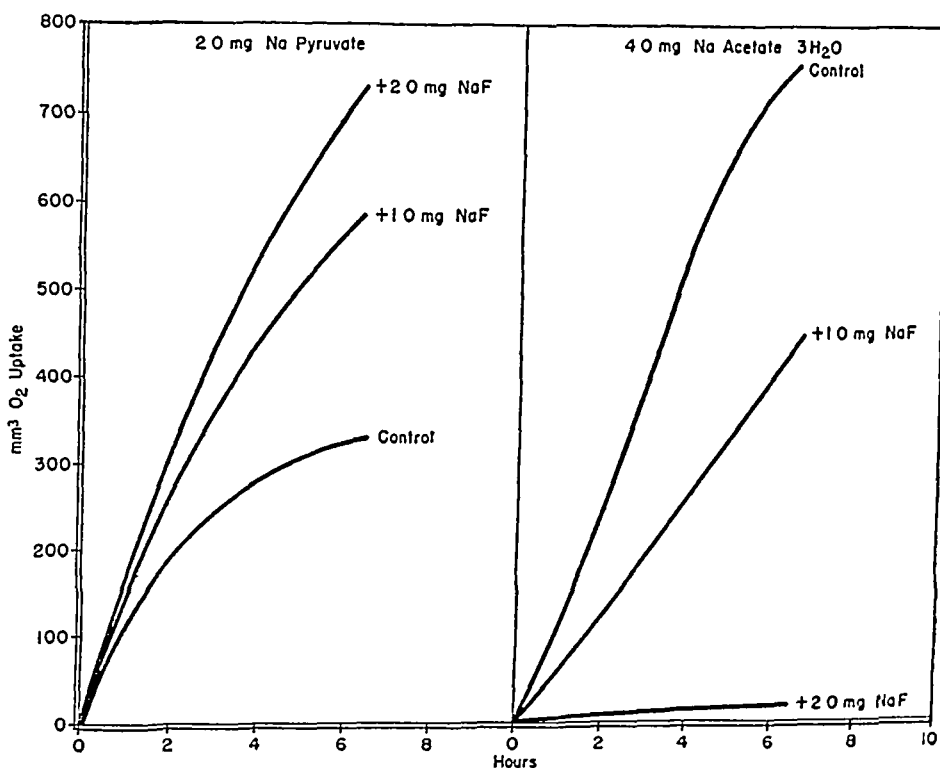


FIG 2 THE EFFECT OF DIFFERENT AMOUNTS OF NaF AT pH 6.0 ON THE OXIDATION OF PYRUVATE AND ACETATE BY MYCOBACTERIUM BCG

The respective control uptakes, with and without fluoride, have been subtracted

indicates that the fluoride effect is not due to the removal of Ca ions. Evidence that fluoride is accelerating an oxidative reaction in the cell is seen in the fact that the R Q, which varies between 0.75 and 0.80 at pH 6.0, is increased to 0.85



to 0.90 in the presence of fluoride. It is possible that carbohydrate oxidation is accelerated, or that the oxidation of fat is inhibited.

These possibilities were investigated by adding various carbohydrates and fatty acids to the bacterial suspensions in the presence and absence of fluoride. The

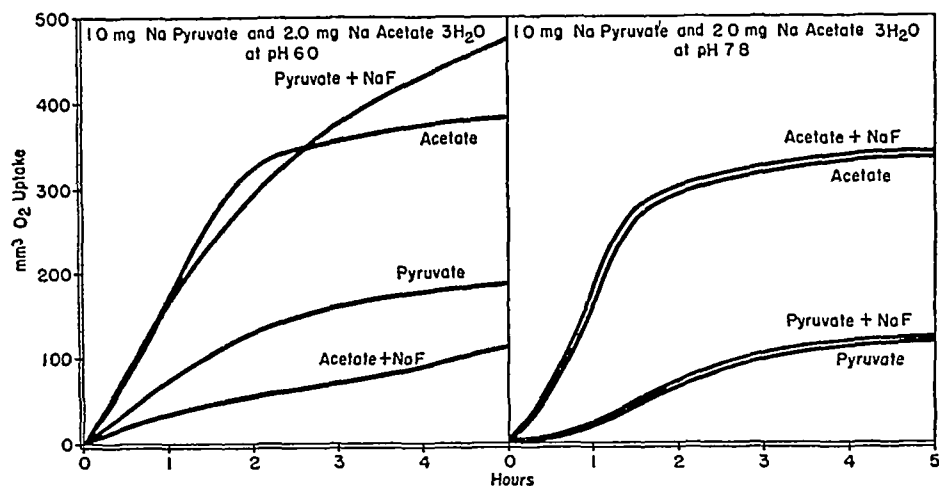


FIG 3 THE EFFECT OF 2.0 MG NaF ON THE OXIDATION OF PYRUVATE AND ACETATE AT pH 6.0 AND 7.8 BY MYCOBACTERIUM BCG

The respective control uptakes, with and without fluoride, have been subtracted

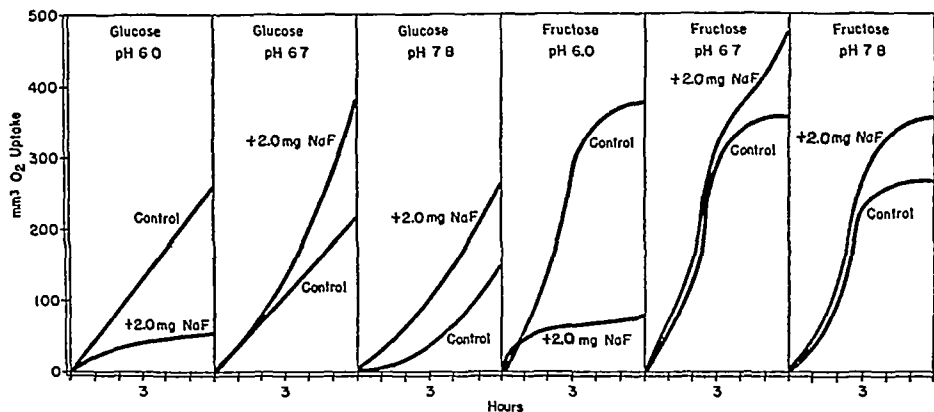


FIG 4 THE EFFECT OF 2.0 MG NaF ON THE OXIDATION BY MYCOBACTERIUM BCG OF GLUCOSE AND FRUCTOSE AT 3 HYDROGEN ION CONCENTRATIONS

The respective control uptakes, with and without fluoride, have been subtracted

results are shown in figures 2 to 5. At pH 6.0 in the presence of fluoride both the rate of oxidation of pyruvate and the absolute O₂ uptake are increased. The maximum effect occurs with 1.0 to 2.0 mg of fluoride, whereas the maximum effect on the control uptake is obtained with 1.0 mg or less. This may mean that a system more sensitive to fluoride is present in the cell. Under the same conditions, fluoride, depending on its concentration, has no effect or in-

hibits the oxidation of acetate. Pyruvate, therefore, cannot be oxidized to acetate by these bacteria but probably condenses with a metabolite in the cell and then is oxidized. Such a condensation probably also occurs during the oxidation of pyruvate in the absence of fluoride, since the oxygen uptake stops when 1 to 2 atoms of oxygen are taken up. If acetate were an intermediate product, the oxidation would proceed to completion because acetate is oxidized under the same conditions to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Decarboxylation, however, accompanies the oxidation of pyruvate because the R Q rises to 1.1 to 1.2 in the absence of, and to 0.96 to 1.1 in the presence of, fluoride.

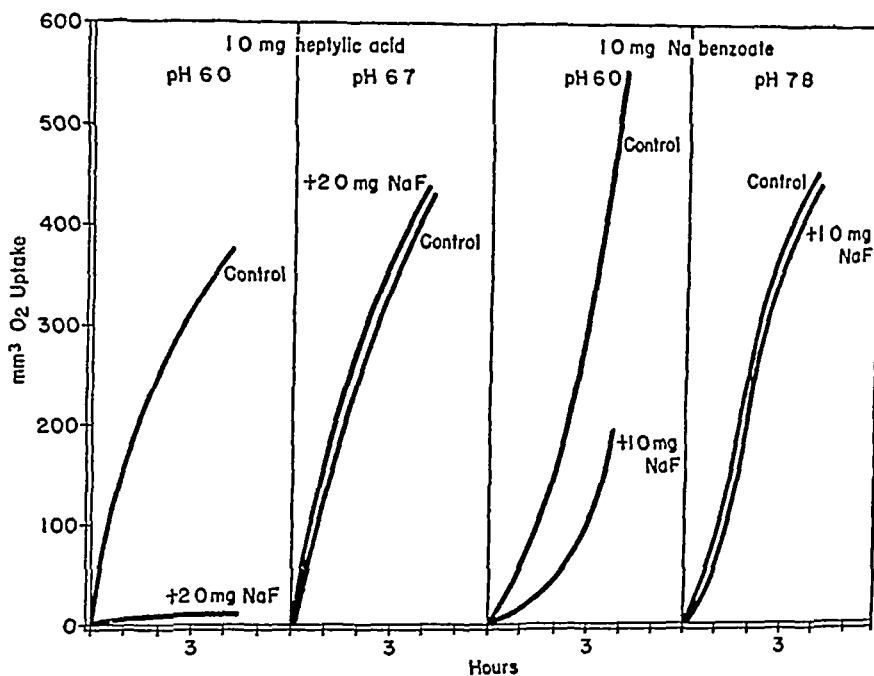


FIG 5 THE EFFECT OF NaF ON THE OXIDATION BY MYCOBACTERIUM BCG OF HEPTYLIC AND BENZOIC ACIDS AT DIFFERENT HYDROGEN ION CONCENTRATIONS

The respective control uptakes, with and without fluoride, have been subtracted

At pH 6.0 the oxidation of *n*-heptylic, oleic, and benzoic acids is inhibited by fluoride. The oxidation of lactate is accelerated in the same way as that of pyruvate, probably because it is readily oxidized to pyruvate. At pH 6.0 the oxidation of glucose, fructose, and trehalose is inhibited by fluoride, but at pH 6.7 or 7.8 a moderate acceleration occurs.

The effect of various concentrations of fluoride on the rate of growth of *Mycobacterium BCG* was investigated using tween medium (Dubos *et al*, 1946) buffered at pH 7.0 and pH 6.0. The effect of adding 50 mg per cent lactate to these media was also investigated. The inoculum for each tube containing 10 ml of medium consisted of 0.5 ml of a 1 to 2 dilution of a 48-hour culture grown in tween medium at pH 7.0 and washed and resuspended in medium of the appropriate

pH Growth was followed nephelometrically by means of the Evelyn photo-electric colorimeter using a 660 filter. At pH 7.0 the growth rate is unaffected by fluoride, or lactate, or a combination of both. At pH 6.0 a different picture appears. The control rate of growth is considerably less than at pH 7.0. In addition, the growth rate is further diminished by 5.0 and 10.0 mg per cent NaF, and this inhibitory effect on growth is potentiated in the presence of 50 mg per cent lactate. This concentration of lactate is not inhibitory in the absence of

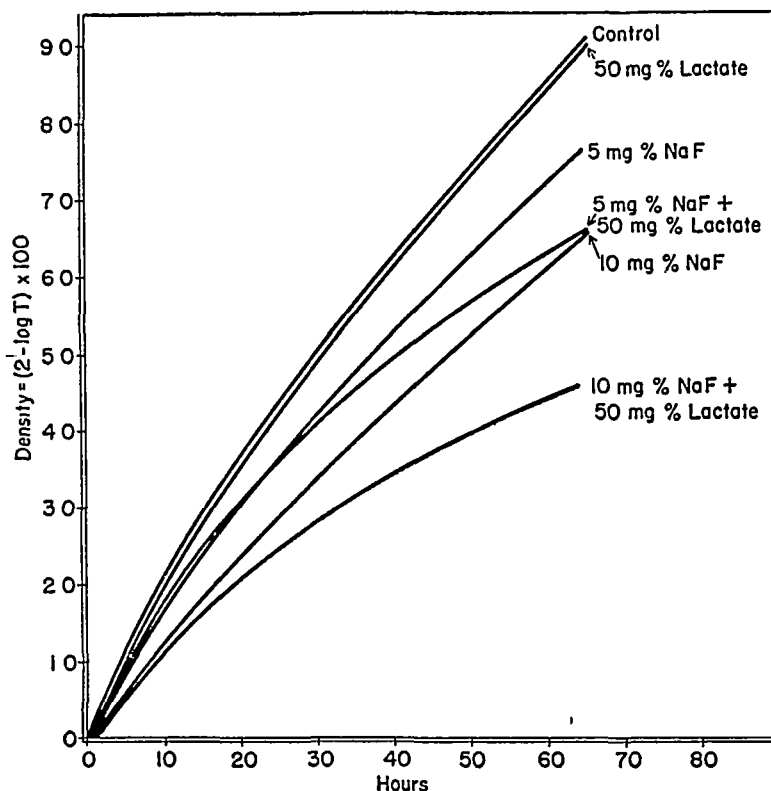


FIG. 6 THE EFFECT OF NaF WITH AND WITHOUT LACTATE ON THE GROWTH OF MYCOBACTERIUM BCG IN DUBOS MEDIUM AT pH 6.0

fluoride. These facts are shown in figure 6. The pH remained constant throughout the experiments. No inhibition of growth was observed with 1.0 mg per cent NaF either in the presence or absence of lactate.

#### SUMMARY

The oxygen uptake of washed suspensions of *Mycobacterium* BCG is greatly increased by sodium fluoride. The amount of the increase depends on the relative concentrations of fluoride and bacteria and upon the hydrogen ion concentration.

The effect is greatest at pH 6.0, intermediate at pH 6.7, and least at pH 7.8

At pH 6.0 the increased uptake is accompanied by a rise in R.Q.

At pH 6.0 the oxidation of pyruvic acid and lactic acid is increased by fluoride, both in rate and amount. The effect diminishes at lower hydrogen ion concentrations.

At pH 6.7 and 7.8 the oxidation of glucose, fructose, and trehalose is increased by fluoride. At pH 6.0 fluoride inhibits the oxidation of these sugars.

At pH 6.0 the oxidation of acetic, heptylic, oleic, and benzoic acids is inhibited by fluoride. At lower hydrogen ion concentrations fluoride has no effect.

At pH 6.0, 5 to 10 mg per cent fluoride inhibits growth, and the presence of lactate increases the inhibition. At pH 7.0 there was no inhibition by fluoride either in the presence or absence of lactate.

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# THE NUTRITIONAL REQUIREMENTS OF *LACTOBACILLUS* *PENTOSUS* 124-2<sup>1</sup>

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*Lactobacillus pentosus* 124-2 is a homolactic bacterium which on hexoses produces almost exclusively lactic acid. It also ferments pentoses, and then necessarily produces other products than lactic acid—mainly acetic acid (Peterson *et al*, 1922, Fred *et al*, 1921). It is a very sturdy organism, since it readily ferments such unfavorable sugar-containing materials as acid hydrolyzates of wood, sawdust, and sulfite waste liquor (Marten *et al*, 1927, Allgeier *et al*, 1929, Leonard *et al*, 1948). Its versatility and vigor make it an organism of possible industrial use, hence a study of its nutritional requirements seemed desirable. It is closely related to the organism that is used for vitamin and amino acid assays, *Lactobacillus arabinosus* 17-5, but on the whole appears to be more vigorous than the latter.

There are but few reports in the literature concerning the nutritional requirements of this organism. In two papers Snell, Strong, and Peterson (1938, 1939) demonstrated the requirement of *L. pentosus* 124-2 for pantothenic acid. Snell and Strong (1939) reported that the organism did not need riboflavin, in fact synthesized it. *Para*-aminobenzoic acid was reported as an essential metabolite by Snell and Mitchell (1942-43). These same authors (1941) reported that the rate of growth was dependent on adenine, but in the presence of uracil part of this effect was nullified. Recently, Dunn and coworkers (1947, Shankman *et al*, 1947) investigated the vitamin and amino acid requirements of 23 lactic acid organisms, including *L. pentosus* 124-2. Pantothenic acid, nicotinic acid, and biotin were reported to be essential vitamins. When one amino acid at a time was omitted, they found valine, leucine, isoleucine, cysteine, and glutamic acid to be indispensable to growth as measured by acid production.

## EXPERIMENTAL METHODS AND RESULTS

**Culture and inoculum** The organism used in these studies was *Lactobacillus pentosus* 124-2. The stock culture was carried as a stab in a medium consisting of 0.5 per cent Difco yeast extract, 0.5 per cent glucose, 0.5 per cent sodium acetate, 2 per cent agar, and 0.05 ml of mineral salt solutions A and B per tube (table 1).

The inoculum was prepared by transferring cells from a stab culture to 10 ml of medium A (table 1). After 24 hours the culture was centrifuged and the cells

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TABLE 1

*Media*

<b>Medium A (Inoculum medium)</b>	
Glucose	1%
Sodium acetate	1%
Difco yeast extract	0.5%
Salt solutions A and B*	0.5 ml of each per tube
<b>Medium B (Hydrolyzed casein medium)</b>	
Glucose	1.0%
Sodium acetate	1.0%
Casein, acid-hydrolyzed	0.5%
L-Cystine	10.0 mg %
DL-Tryptophan	2.5 mg %
Adenine sulfate	2.0 mg %
Calcium pantothenate	0.05 mg %
Nicotinic acid	0.25 mg %
Para-aminobenzoic acid	0.025 mg %
Biotin	5.0 millimicrograms per tube
Salt solutions A and B*	0.5 ml of each per tube
pH 6.0	
<b>Medium C (Amino acid synthetic medium)</b>	
Glucose	1.0%
Sodium acetate	1.0%
DL-Methionine	2.0 mg % of each per tube
DL-Tryptophan	
L-Tyrosine	
DL-Aspartic acid	
L-Histidine HCl	
L-Arginine HCl	
DL-Serine	
L-Proline	
L-Hydroxyproline	
DL-Lysine HCl	
L-Cystine	
DL-Threonine	
DL-Alanine	
DL-Valine	
DL-Leucine	
DL-Isoleucine	
DL-Phenylalanine	
DL-Glutamic acid	
Glycine	
Adenine sulfate	2.0 mg %
Calcium pantothenate	0.05 mg %
Nicotinic acid	0.25 mg %
Para aminobenzoic acid	0.025 mg %
Biotin	5.0 millimicrograms per tube
Salt solutions A and B*	0.5 ml of each per tube
pH 6.0	

\* Salt solution A: 25 g  $K_2HPO_4$  and 25 g  $KH_2PO_4$  in 250 ml water. Salt solution B: 10 g  $MgSO_4 \cdot 7H_2O$ , 0.5 g  $NaCl$ , 0.5 g  $FeSO_4 \cdot 7H_2O$ , and 0.5 g  $MnSO_4 \cdot 4H_2O$  in 250 ml water.

were resuspended in 10 ml of sterile water. The Evelyn colorimeter reading (percentage of light transmission) of this suspension with a 660 m $\mu$  filter, against a water blank at 100, was 38. Five-tenths ml of this suspension were transferred to another 10 ml of sterile water, and one drop of this second suspension was used to inoculate each tube in the assay series.

**Media** The composition of the basal medium, B, is given in table 1. The hydrolyzed casein was prepared by autoclaving 50 g of Labco vitamin-free casein with 500 ml of 12 N sulfuric acid for 18 hours at 15 pounds pressure. The sulfate was removed by precipitation with 640 g of Ba(OH)<sub>2</sub> · 8H<sub>2</sub>O in 400 ml of boiling water. The precipitate was washed with 500 ml of boiling water, filtered again, and the filtrates were combined. The combined filtrates were adjusted

TABLE 2  
*Effect of inoculum on rate of growth and vitamin requirements*

INOCULUM*	GROWTH RATE ON MEDIUM B PLUS ALL OTHER KNOWN VITAMINS								BIOTIN OMITTED	PANTOTHE NATE OMITTED	NICOTINIC ACID OMITTED					
	12 hr		24 hr		48 hr		72 hr				72 hr					
	ER†	Acid†	ER	Acid	ER	Acid	ER	Acid			ER	Acid	ER	Acid	ER	Acid
A	60	6 2	35	8 9	30	10 1	25	10 1	90	1 4	85	3 2	82	5 0		
B	88	4 1	39	7 9	33	10 0	30	10 2	93	0 8	90	1 0	92	1 0		
C	90	2 3	42	6 5	34	10 1	30	10 0	92	0 8	94	0 8	92	1 0		
D	92	1 6	81	4 0	56	7 2	35	9 1	96	0 4	98	0 5	100	0 6		
Uninoculated							100	0 4								

\* One drop of each of the following inocula was used.

Inoculum A no dilution of the suspension from 24-hr culture.

Inoculum B 0.5 ml added to 10 ml sterile water.

Inoculum C one drop added to 10 ml sterile water.

Inoculum D 1 ml added to 10 ml sterile water, and then 0.1 ml of this suspension added to another 10 ml sterile water.

† ER = Evelyn colorimeter reading.

Acid = ml N/10 NaOH required to titrate 10 ml medium.

to pH 3 with NaOH, and the volume was reduced to 1 liter by vacuum distillation. Five g of norit A were then added, and the mixture was stirred for one-half hour and filtered through filter-cel. The filtrate is designated as a 5 per cent solution of hydrolyzed casein. The medium was adjusted to pH 6.0 with NaOH.

**Procedure** Five ml per tube of double strength medium B were used. The material to be tested was omitted from the basal medium, added separately to each tube (18 by 150 mm), and water was added to make 10 ml. The tubes were plugged with cotton and autoclaved 15 minutes at 15 pounds pressure. When cool, the tubes were inoculated with one drop of the inoculum described above, and incubated at 37°C. The titrations were made to the phenol red end point.

**Effect of inoculum and initial pH** Concurrently with the determination of the nutritional requirements of *L. pentosus* 124-2, the effects of inoculum and initial pH were investigated. Table 2 shows the effect of different inocula on the

vitamin requirements and rate of growth on medium B plus all other known vitamins. Inoculum B was chosen for all further work since, on a complete medium, it allowed fairly rapid growth and gave low blanks in the absence of a specific metabolite.

The initial pH of the medium influenced the initiation of growth, but from pH 5.5 to 7.0 did not markedly affect the final turbidity at 72 hours. This effect was the same on both medium B and medium C. Figure 1 shows the effect of initial pH on the turbidity of the cultures on the two media at 30 hours.

*Vitamin requirements* Biotin, nicotinic acid, and pantothenic acid were found to be essential. This is in agreement with the findings of Snell, Strong, and

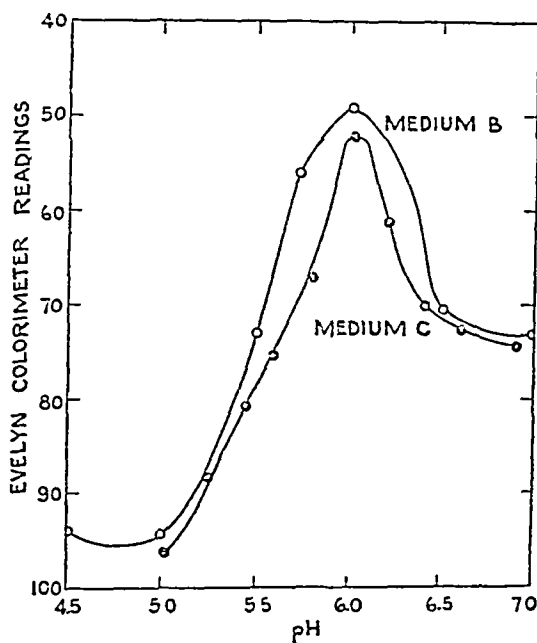


FIG 1 EFFECT OF INITIAL pH ON INITIATION OF GROWTH OF *LACTOBACILLUS PENTOSUS* 124-2 (30 HOURS)

Peterson (1938, 1939) and Shankman *et al* (1947). Titrations and Evelyn colorimeter readings at the end of 72 hours, when graded amounts of each of these vitamins were added, are given in table 3.

If specially prepared casein was used (Lampen and Peterson, 1941), a requirement for *p*-aminobenzoic acid could be demonstrated. When no *p*-aminobenzoic acid was present, the Evelyn colorimeter reading was 88 and the titration of 10 ml of medium with  $\times/10$  NaOH was 2.3. Maximum growth occurred in the presence of 1 millimicrogram of *p*-aminobenzoic acid. This is in agreement with the findings of Snell and Mitchell (1942-43), but Shankman *et al* (1947) did not find *p*-aminobenzoic acid to be an essential metabolite.

*Effect of purines and pyrimidines* Fairly good growth can be obtained in the complete absence of purines and pyrimidines, as is shown in table 4. However, adenine was stimulatory both on the growth rate and on final growth. Adenine



acid was no better than adenine. Guanine seemed to be slightly inhibitory. Cytosine and guanylic acid may be somewhat stimulatory.

*Effect of other materials on the growth rate.* Although the growth rate of *L. pentosus* 124-2 on the hydrolyzed casein medium (medium B, table 1) was not so rapid as that on "natural" medium (medium A, table 1), after 72 hours cell

TABLE 3

*Response of L. pentosus 124-2 to biotin, pantothenic acid, and nicotinic acid*

VITAMIN	AMOUNT	EVELIN READINGS	ML N/10 NaOH/10 ML MEDIUM
Biotin	<i>millimicrograms</i>		
	0	92	1 0
	0 05	85	1 7
	0 1	79	2 9
	0 2	67	5 0
	0 3	58	6 6
	0 4	54	7 8
	0 5	49	8 5
	0 6	48	9 6
	1 0	44	9 7
	2 0	31	9 6
	20 0	28	9 8
Pantothenic acid	<i>micrograms</i>		
	0	90	1 0
	0 05	65	6 4
	0 1	52	7 3
	0 15	47	9 1
	0 2	43	9 0
	0 6	28	9 3
	1 0	28	9 6
Nicotinic acid	0	93	0 8
	0 2	65	2 9
	0 4	39	4 7
	0 6	29	6 4
	0 8	30	6 9
	1 0	28	7 8
	2 0	28	9 4

growth (turbidity) and acid production on the two media were nearly equal, as can be seen in table 4.

Various known stimulatory substances and preparations were tried in an attempt to increase the growth rate on the hydrolyzed casein medium. Glutathione (Seitz-filter-sterilized), glutamine (Seitz-filter-sterilized), and streptogenin (an enzymatic digest of casein) and a concentrate<sup>2</sup> prepared from casein by the method of Sprince and Woolley (1945) were without effect. The whey prepara-

<sup>2</sup> This concentrate was supplied by G. M. Shull.

tion of Scott, Norris, and Heuser (1946), the peptone preparation of Teply and Elvehjem (1945), and up to 0.1 mg per cent of Wilson's liver B preparations

TABLE 4  
Growth rate of *L. pentosus* 124-2 on various media

MEDIA	ADDITION	AMOUNT TUBE	EVELYN READINGS				
			14 hr	16 hr	18 hr	35 hr	42 hr
Medium B (less adenine)	None		97		95	39	35
	Adenine	0.2 mg	97		85	32	30
	Adenylic acid	0.2 mg	96		86	35	30
	Guanine	0.2 mg					63
	{ Adenine	0.2 mg					17
	{ Guanine	0.2 mg					17
	{ Adenine	0.2 mg					
	{ Cytosine	0.2 mg	79	72	67		23
	Guanylic acid	0.2 mg					
	{ Adenine	+ glutamine	79	71	67		29
	{ Cytosine						
	{ Guanylic acid						
	{ Adenine	+ glutathione	83	71	70		30
	{ Cytosine						
	{ Guanylic acid						
	Adenine	0.2 mg					
	Tryptic digest of casein	2.0 ml	94		88		30
	Adenine	0.2 mg					
	Streptogenin concentrate	0.1 ml	93		87		31
	Adenine	0.2 mg					
	Cytosine	0.2 mg					
	Guanylic acid	0.2 mg	71	65	59		30
	Malt sprouts prep *	0.05 ml					
	Adenine	0.2 mg					
	Wilson's liver B	1.0 mg	80	73	70		30
Medium C (contains adenine)	None		98		89	10	30
	{ Cytosine	0.2 mg					
	{ Guanylic acid	0.2 mg	82	75	70		30
	{ Cytosine	0.2 mg					
	{ Guanylic acid	0.2 mg	75	67	60		31
Medium A	Malt sprouts prep *	0.05 ml					
	None		62	51	42		27
	Wilson's liver B	1.0 mg	60	50	42		26
	Malt sprouts prep *	0.05 ml	53	48	43		25

\* A 20 per cent suspension of malt sprouts in water was incubated overnight at 15°C, pressed out through cheesecloth, and the extract stored under toluene. One ml of the extract is equivalent to 0.2 g of malt sprouts.

caused no stimulation of the growth rate. Of the natural material tried, an aqueous extract of malt sprouts was most effective (table 1). Pan, Peterson, and Johnson (1940) have reported marked stimulation of a lactic fermentation

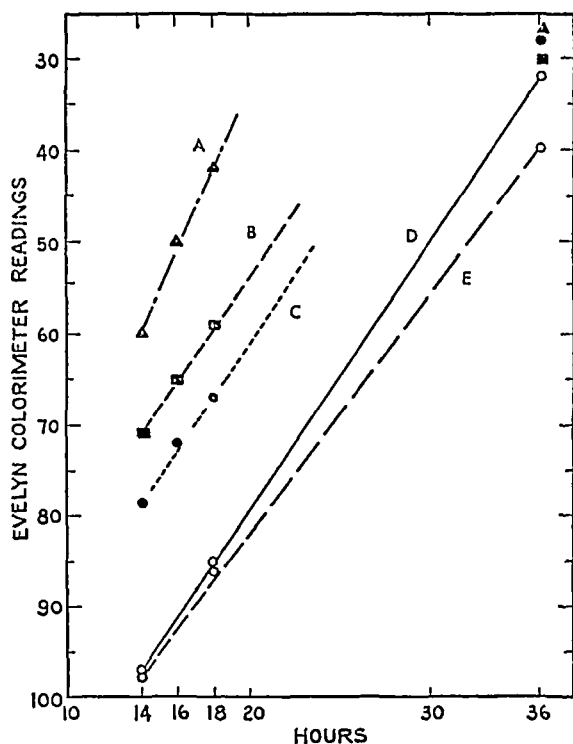


FIG 2 GROWTH RATE OF LACTOBACILLUS PENTOSUS 124-2 ON VARIOUS MEDIA

A, medium A, B, medium B plus cytosine, guanylic acid, and malt sprouts preparation, C, medium B plus cytosine and guanylic acid, D, medium B

TABLE 5

*Amino acid requirements of Lactobacillus pentosus 124-2*

AMINO ACID OMITTED	EVELYN READING	ML N/10NaOH/10 ml medium
	72 hours	
Glycine	30	9 2
DL-Methionine	31	9 3
DL Tryptophan	54	10 1
L-Tyrosine	46	10 0
DL-Aspartic acid	52	9 8
L Histidine HCl	34	10 2
L-Arginine HCl	38	10 1
DL Serine	30	10 2
L-Hydroxyproline	28	10 3
L-Proline	29	10 1
DL Lysine HCl	35	10 3
L-Cystine	76	5 1
DL-Threonine	60	6 8
DL-Alanine	75	4 5
DL-Valine	94	1 2
DL Leucine	93	1 7
DL-Isoleucine	90	1 7
DL-Phenylalanine	94	1 8
DL-Glutamic acid	99	1 4
None	30	10 0

of glucose or molasses by the addition of unheated malt sprouts Figure 2 shows the growth rate on a number of the media given in table 4

The best combinations for maximum growth rate on the hydrolyzed casein medium were also tried on the amino acid medium and, as is shown in table 4, gave nearly identical results

TABLE 6  
*Amino acid requirements of L pentosus 124-2*

MEDIUM	72 hours	
	Evelyn readings	ml N/10 NaOH/10 ml medium
Basal*	92	1 3
Basal + { tryptophan arginine	78	3 8
Basal + { aspartic acid		
Basal + { lysine		
Basal + { histidine		
Basal + { tyrosine		
+ serine	76	4 2
+ proline	80	3 3
+ hydroxyproline	80	3 3
+ methionine	71	4 7
+ serine + proline	68	4 4
+ serine + hydroxyproline	76	4 1
+ serine + methionine	46	9 9
+ { serine proline	76	4 0
+ { hydroxyproline		
+ { serine proline		
+ { methionine	33	9 8
+ { proline		
+ { hydroxyproline		
+ { methionine	81	3 3
+ { serine		
+ { proline		
+ { hydroxyproline	45	8 4
+ { methionine		

\* Basal contains 2 mg each per tube of alanine, valine, leucine, isoleucine, glutamic acid, phenylalanine, threonine, and cystine

*Amino acid requirements* The synthetic medium used for testing amino acid requirements was medium C (table 1) When only one amino acid at a time was omitted from the mixture, the turbidities and titrations given in table 5 were obtained From these results, alanine, valine, leucine, isoleucine, glutamic acid, and phenylalanine were definitely essential, and threonine and cystine were probably also necessary The same results were obtained even in the presence of 10 micrograms per ml of pyridoxal Therefore, under the condi-

tions used here, the amino acid requirements of *L. pentosus* 124-2 are not affected by the presence or absence of pyridoxal, as are the amino acid requirements of *L. arabinosus* 17-5 (Lyman *et al*, 1947). For this reason, this organism may prove to be more satisfactory than *L. arabinosus* 17-5 for amino acid assays.

These results are not entirely in agreement with Dunn *et al* (1947), who obtained maximum titrations even when phenylalanine was omitted. However, their inoculum was much heavier, being diluted only 3 times from a 24-hour culture, whereas the inoculum used here was diluted 21 times. If the concentration of inoculum used by Dunn *et al* (1947) was used and phenylalanine omitted, a titration of 6.2 ml was obtained. This is not maximal, but shows that the greater part of the difference for the discrepancy between the results of Dunn *et al* (1947) and the results obtained here is due to the inoculum.

However, the nitrogen requirements of *L. pentosus* 124-2 have not been solved unless the organism is able to grow in the presence of only these amino acids. When this was tried (table 6), little growth was obtained in the presence of only those amino acids previously found to be essential, even when the deficiency of nitrogen compared to the medium with all the amino acids was made up by the addition of dihydrogen ammonium phosphate. In table 6 are given only the more conclusive results from the hundreds of combinations tried. It can be seen that really good growth and maximum acid production were obtained only in the presence of all the amino acids tried except glycine and hydroxyproline. As can be seen in table 6, hydroxyproline was never stimulatory, and in three of the five combinations shown in the table was definitely inhibitory.

#### SUMMARY

The nutritional requirements of *Lactobacillus pentosus* 124-2 were investigated. Biotin, pantothenic acid, and nicotinic acid are needed to satisfy its vitamin requirements.

No chemically defined medium gave so rapid growth as did a natural medium. One of the best media, hydrolyzed casein, required some addition, but glutathione, glutamine, streptogenin, Wilson's liver B, a whey preparation, and a peptone preparation had no effect. An aqueous extract of malt sprouts caused the most marked stimulation. The nature of the stimulatory factor is unknown.

The single omission of valine, leucine, isoleucine, glutamic acid, and phenylalanine from an adequate medium containing twenty amino acids resulted in the prevention of growth of *L. pentosus* 124-2. Maximum growth did not occur on the single omission of cystine, threonine, or alanine. However, it was necessary for tryptophan, arginine, aspartic acid, lysine, histidine, tyrosine, serine, proline, and methionine also to be present in the medium if growth near that possible on a "natural" medium was to be obtained.

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# METABOLISM OF BIOTIN AND OXYBIOTIN BY *LACTOBACILLUS PENTOSUS* 124-2<sup>1</sup>

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The demonstration of biotin activity for microorganisms of such analogs of biotin as oxybiotin (Hofmann *et al*, 1945, Winnick *et al*, 1945, Pilgrim *et al*, 1945, Rubin *et al*, 1945b, Duschinsky *et al*, 1945), and desthiobiotin (Melville *et al*, 1943, Dittmer *et al*, 1944, Lilly and Leonian, 1944, Leonian and Lilly, 1945, Tatum, 1945, Stokes and Gunness, 1945) has aroused an interest in the manner in which these compounds function in cell metabolism. The role of desthiobiotin has been fairly well elucidated. Desthiobiotin is as potent as biotin for a number of microorganisms, mostly yeasts and fungi (Melville *et al*, 1943, Dittmer *et al*, 1944, Lilly and Leonian, 1944, Leonian and Lilly, 1945, Tatum, 1945, Stokes and Gunness, 1945), and is converted into biotin by these organisms (Dittmer *et al*, 1944, Tatum, 1945, Leonian and Lilly, 1945, Stokes and Gunness, 1945). It is probably an intermediate in the biosynthesis of biotin, since an X-ray mutant of *Penicillium chrysogenum*, strain 62078, could synthesize desthiobiotin but could not convert it to biotin (Tatum, 1945), whereas the original strain could synthesize biotin and could also convert desthiobiotin to biotin. Desthiobiotin acts as an inhibitor to both biotin (Dittmer and du Vigneaud, 1944, Lilly and Leonian, 1944, Tatum, 1945, Rubin *et al*, 1945a) and oxybiotin (Rubin *et al*, 1945b) for *Lactobacillus casei*, but has no effect on the growth of *Lactobacillus arabinosus* and *Rhizobium trifolii* (Lilly and Leonian, 1944) either alone or in the presence of biotin.

The role of oxybiotin in the metabolism of microorganisms was unsettled until recently. Rubin *et al* (1945b) reported data obtained by utilizing the differences in activity of oxybiotin and biotin for *Saccharomyces cerevisiae*, which indicated that *O*-heterobiotin (oxybiotin) was converted into biotin or some other compound of comparable activity for *S. cerevisiae* by the organism during growth. Hofmann and coworkers (Hofmann, 1945, Axelrod *et al*, 1947), using the method of Rubin *et al* (1945b) as well as three differential assay procedures developed by them (Hofmann and Winnick, 1945, Axelrod *et al*, 1946, Hofmann *et al*, 1947), seem to have solved the problem by showing conclusively that yeast assimilates oxybiotin as such, and does not convert it into biotin. The assimilation of an analog not found in nature, and the apparent use of it by the cell in place of the natural compound in its metabolism, is a unique phenomenon.

Whether or not any part of a given vitamin is destroyed in metabolism has not been investigated extensively. Leonian and Lilly (1945) were unable to

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

recover all the added biotin and desthiobiotin from the cell-free medium or cells of several yeasts and molds that were given a great excess of these compounds. In investigating the metabolism of oxybiotin by yeast, Axelrod *et al* (1947) recovered more than 70 per cent of the oxybiotin from the yeast cells. McIlwain and Hughes (1944) obtained a recovery of from only 2 to 5 per cent of the supplied pantothenic acid from cultures of hemolytic streptococci that were given an excess of the vitamin.

A more extreme example with a somewhat different aspect is the microbial utilization of vitamins as the sole source of carbon. Metzger (1947) reported the decomposition of pantothenate, present as the only carbon source, by several *Pseudomonas* species. Likewise, Koser and Baird (1944) reported the destruction of nicotinic acid, when this was present as the sole source of carbon, by

TABLE 1  
*Media for Lactobacillus pentosus 124-2*

Glucose	1.0%
Sodium acetate	1.0%
Casein, acid-hydrolyzed	0.5%
L-Cystine	10 mg %
DL-Tryptophan	2.5 mg %
Adenine sulfate	2.0 mg %
Calcium pantothenate	0.05 mg %
Nicotinic acid	0.25 mg %
p-Aminobenzoic acid	0.025 mg %
Biotin	5.0 millimicrograms per tube
Salt solutions A and B*	0.5 ml of each per tube
pH 6.0	

\* Salt solution A: 25 g  $K_2HPO_4$  and 25 g  $KH_2PO_4$  in 250 ml water.

Salt solution B: 10 g  $MgSO_4 \cdot 7H_2O$ , 0.5 g NaCl, 0.5 g  $FeSO_4 \cdot 7H_2O$ , and 0.5 g  $MnSO_4 \cdot 4H_2O$  in 250 ml water.

*Pseudomonas fluorescens* and *Serratia marcescens*. Foster (1944) described an organism capable of oxidizing riboflavin to lumichrome, and Mirick (1943) isolated a soil organism capable of oxidizing p-aminobenzoic acid. Since vitamins have been shown to serve as sole carbon substrates, it would not be unreasonable to find that excess of an "essential" vitamin could also be metabolized.

#### EXPERIMENTAL METHODS AND RESULTS

**Cultures and media.** The organisms used in this study were *Lactobacillus pentosus* 124-2 and *Lactobacillus arabinosus* 17-5 (ATCC no. 8014). The stock cultures were carried as stabs in a medium consisting of 0.5 per cent Difco yeast extract, 0.5 per cent glucose, 0.5 per cent sodium acetate, 2 per cent agar, and 0.05 ml of mineral salt solutions A and B per tube (table 1). The inoculum was prepared by transferring cells from a stab culture to a medium consisting of 0.5 per cent Difco yeast extract, 1.0 per cent glucose, 1.0 per cent sodium acetate,



and 0.05 ml of mineral salt solutions A and B per tube (table 1). After 24 hours the culture was centrifuged, and the cells were resuspended in 10 ml of sterile water. The Evelyn colorimeter reading (percentage of light transmission) of this suspension with a 660-m $\mu$  filter, against a water blank at 100, was 38. Five-tenths ml of this suspension were transferred to another 10 ml of sterile water, and one drop of this second suspension was used to inoculate each tube in the assay series.

The composition of the basal medium for *L. pentosus* 124-2 is given in table 1. The hydrolyzed casein was prepared by autoclaving 50 g of Labco vitamin-free casein with 500 ml of 12 N sulfuric acid for 18 hours at 15 pounds pressure. The sulfate was removed by precipitation with 640 g of Ba(OH)<sub>2</sub> · 8H<sub>2</sub>O in 400 ml of boiling water. The precipitate was washed with 500 ml of boiling water, filtered again, and the filtrates were combined. The combined filtrates were adjusted to pH 3 with NaOH, and the volume was reduced to 1 liter by vacuum distillation. Five g of norit A were then added, and the mixture was stirred for one-half hour and filtered through filter cel. The filtrate is designated as a 5 per cent solution of hydrolyzed casein.

Five ml per tube of double-strength medium were used. The biotin-containing samples were added to the tubes, and distilled water was added to make 10 ml. The tubes were plugged with cotton and autoclaved for 15 minutes at 15 pounds pressure. When cool, the tubes were inoculated with one drop of the inoculum described above and incubated at 37 C. After 72 hours the tubes were read in an Evelyn photoelectric colorimeter against a 660-m $\mu$  filter, and then titrated to the phenol red end point with N/10 NaOH.

*Hydrolysis of cells for assay.* Cells were hydrolyzed in 2 N H<sub>2</sub>SO<sub>4</sub> in the autoclave at 15 pounds pressure for 2 hours.<sup>2</sup> One ml of acid was used for each 50 mg or less of cell weight. The hydrolyzate was neutralized with barium hydroxide, and the sulfate removed by filtering. The sulfate was thoroughly washed, and the washings were added to the filtrate. The filtrates were made up to an appropriate volume and used directly in the assay. Recoveries of added biotin ranged from 90 to 103 per cent.

The medium, after removal of the cells by centrifugation, was autoclaved at 15 pounds pressure for 15 minutes. It was then made up to an appropriate volume and assayed without further treatment. Recovery of biotin from uninoculated medium subjected to the same treatment as the growing cultures was 97 to 102 per cent.

*Response of L. pentosus to biotin, oxybiotin, and desthiobiotin.* Typical titrimetric and turbidimetric curves for the response of *L. pentosus* to biotin are shown in figure 1. The relationship of oxybiotin and desthiobiotin in the nutrition of *Lactobacillus pentosus* 124-2 was investigated, and the results are shown in figure 2. Oxybiotin (DL) was found to have 50 per cent of the activity of D-biotin, therefore, if only one of the optical isomers is active, as is the case with DL-biotin, then oxybiotin possesses the same activity as biotin for this organism.

<sup>2</sup> This concentration of acid and procedure for hydrolysis gave good recovery of added biotin and highest values on cell hydrolyzates.

This effect has also been reported with *L arabinosus* (Hofmann *et al*, 1945, Winnick *et al*, 1945, Pilgrim *et al*, 1945, Rubin *et al*, 1945b)

*L pentosus* again resembles *L arabinosus* (Lilly and Leonian, 1944) in its

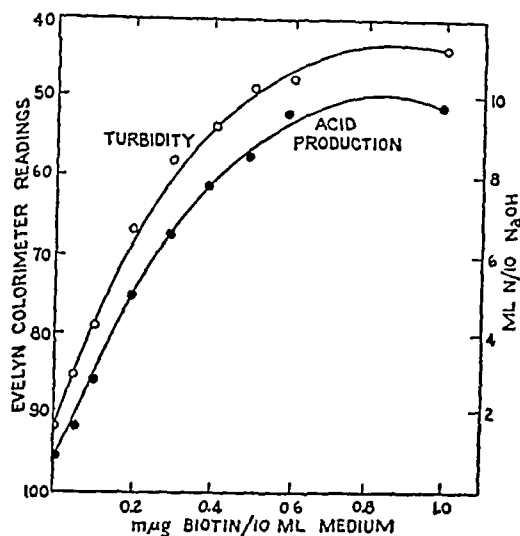


FIG 1 RESPONSE OF LACTOBACILLUS PENTOSUS 124-2 TO BIOTIN

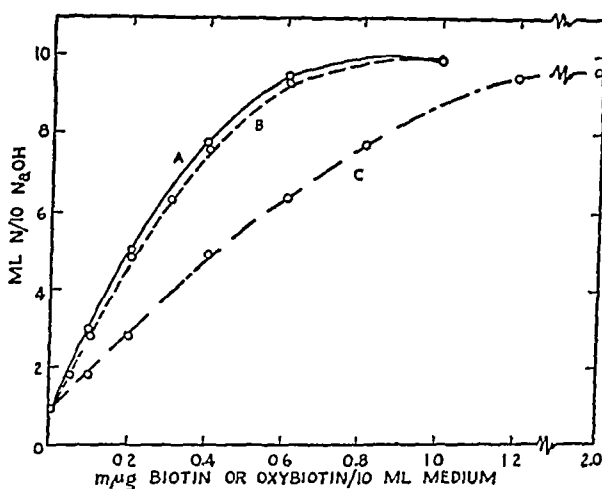


FIG 2 RESPONSE OF LACTOBACILLUS PENTOSUS 124-2 TO DESTHIOBIOTIN AND OXYBIOTIN

A, biotin or biotin plus 2 μg desthiobiotin per tube, B, DL oxybiotin, C, DL oxybiotin

response to desthiobiotin. As can be seen from figure 2, up to 2 micrograms per tube of desthiobiotin elicit no measurable response from this organism.

Two differential assays for biotin and oxybiotin were used. The method of Hofmann and Winnick (1945), using potassium permanganate to oxidize the biotin to biotin sulfone but having no effect on oxybiotin, was used to measure oxybiotin in the presence of biotin with *L pentosus* as the test organism. Figure 3 shows typical curves obtained with known amounts of biotin and oxybiotin.

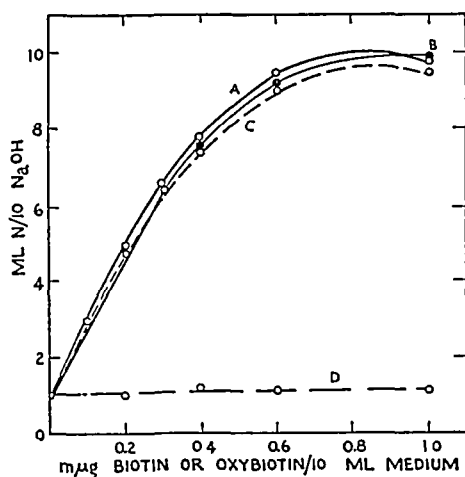


FIG 3 RESPONSE OF *LACTOBACILLUS PENTOSUS* 124-2 TO PERMANGANATE-TREATED BIOTIN AND OXYBIOTIN

A, oxybiotin, B, oxybiotin in the presence of 1 mμg biotin per tube, permanganate-treated, C, oxybiotin, permanganate-treated, D, biotin, permanganate-treated

TABLE 2

Growth rate of *L. pentosus* 124-2 on biotin and oxybiotin\*

MILLIMICRO GRAMS PER ML	12 HR ER†	18 HR		24 HR		30 HR		42 HR		46 HR		60 HR		72 HR	
		ER	Acid	ER	Acid	ER	Acid	ER	Acid	ER	Acid	ER	Acid	ER	Acid
Biotin‡															
0														98	1 2
0 02	95	81	2 1	81	2 4	77	2 8	75	4 1	72	4 5	71	4 9	68	4 9
0 04	91	75	2 8	73	3 5	67	4 3	64	5 5	55	6 5	57	7 1	51	7 5
0 06	96	76	2 8	67	3 9	61	5 3	57	7 4	52	8 2	48	9 1	48	9 4
0 10	89	66	3 7	60	4 6	52	6 6	47	9 6	42	10 1	41	—	40	10 1
0 20	75	51	5 5	50	5 6	38	9 2	40	10 2	39	—	33	—	33	10 2
0 30	83	47	5 6	42	6 9	38	10 1	37	—	36	—	34	—	32	10 1
0 50	85	48	5 4	40	7 1	36	10 2	38	—	42	—	31	—	32	10 0
1 0	83	49	5 3	38	6 6	38	10 1	37	—	33	—	30	—	30	9 9
5 0	88	48	5 4	39	7 9	37	10 2	37	—	34	—	30	—	31	10 1
10 0	85	47	5 4	38	8 3	42	10 1	36	—	35	—	30	—	30	10 1
50 0	78	44	6 1	39	8 0	38	10 2	35	—	33	—	30	—	30	10 2
Oxybiotin‡															
0 04	93	82	2 6	73	3 5	68	4 5	65	5 6	62	6 4	56	7 2	50	7 2
0 06	91	73	2 9	69	3 6	63	5 0	57	6 9	52	7 9	48	9 1	47	9 2
0 10	87	66	3 6	65	4 4	54	6 4	49	9 0	43	9 6	43	10 2	41	10 2
1 0	86	45	5 2	39	7 2	41	9 8	32	10 5	32	—	33	—	33	10 1
10 0	76	46	6 1	42	7 2	39	10 2	36	—	34	—	31	—	31	10 2
50 0	84	48	5 3	40	7 0	40	10 1	38	—	35	—	30	—	30	9 9

\* Figures are averages of 4 runs

† ER = Evelyn colorimeter reading, Acid = ml N/10 NaOH used to titrate 10 ml of culture

‡ Figures are for the D isomer

Biotin in the presence of oxybiotin was measured by the method of Axelrod, DeWoody, and Hofmann (1946), in which  $\gamma$ -(3,4-urelyenecyclohexyl)butyric acid was used in concentrations that inhibit oxybiotin but do not affect biotin for *L. arabinosus* 17-5 }

In another experiment biotin and oxybiotin were found to be completely additive in the presence of each other in their activity for *L. pentosus* 124-2. Therefore, assays for total activity could be validly expressed as either compound.

*Growth rate of L. pentosus on biotin and oxybiotin* The growth rate as measured by turbidity and acid production on graded and excess amounts of biotin and oxybiotin is shown in table 2. From the data on acid production it can be seen that the rate of production of acid increases with increasing biotin concentration up to 0.3 millimicrograms per ml for the first 24 hours. This amount is three times that necessary for maximum acid production in 72 hours. With 0.3 millimicrograms per ml of biotin the acid production reached a maximum in 30 hours. There is an indication from these data that high levels of biotin affect the rate of acid production to a greater extent than the rate of cell production. For example, at the 0.2-millimicrograms-per-ml level of biotin, acid production was finished at 42 hours, whereas turbidity increased until 60 hours, at the 1.0-millimicrogram-per-ml level of biotin, acid production was finished at 30 hours, but turbidity again increased until 60 hours, at the 10-millimicrograms-per-ml level of biotin this was again the case. The final turbidity reading (i.e., at 72 hours) is not at a maximum with 0.1 millimicrogram per ml as is the case with acid production, 0.2 millimicrograms per ml appear to be sufficient for maximum cell production also.

Also in table 2 is shown the rate of cell growth and acid production for *L. pentosus* on oxybiotin. In no case is there any significant difference in the response, as measured by cell growth or acid production, of this organism to biotin and oxybiotin. This is further proof that oxybiotin is able completely to replace biotin for *L. pentosus*.

*The metabolism of biotin and oxybiotin by L. pentosus* The metabolism of biotin and oxybiotin by means of recoveries from the medium and cells of cultures of *L. pentosus* was investigated. Table 3 shows the recovery of biotin where graded amounts of biotin were employed. Only when the organism was grown on minimal amounts of biotin could anywhere near 100 per cent of the biotin be recovered. It is also noteworthy that biotin was found entirely in the cell portion. As the amount of biotin employed was increased beyond the point just necessary for maximum growth and acid production (i.e., 0.1 millimicrogram per ml), the total recovery of biotin from the cells and the medium fell markedly, and eventually reached only 10 to 15 per cent of that added to the medium. However, at this point, where the recovery markedly decreased, very small amounts of biotin could be recovered from the medium, this amount increasing as the amount of biotin added originally increased. This amount, however, remained a small percentage of the total biotin recovered. Stokes and Gunness (1945) also reported the fact that only small amounts of biotin were left in the medium.

TABLE 3  
*Metabolism of biotin by Lactobacillus pentosus 1242*

RUN NO	MILLIMICROGRAMS BIOTIN PER ML ADDED TO MEDIUM BEFORE INOCU- LATION	EVELYN COLORIMETER READING 72 HR	MILLIMICROGRAMS BIOTIN PER ML OF ORIGINAL CULTURE*			PERCENTAGE OF RECOVERY OF BIOTIN
			In medium	In cells	Total	
1	0 02	66	0	0 02	0 02	100
5		67	0	0 02	0 02	100
1	0 04	50	0	0 044	0 044	110
2		48	0	0 042	0 042	105
5		52	0	0 039	0 039	98
1	0 06	48	0	0 058	0 058	97
2		46	0	0 060	0 060	100
3		48	0	0 054	0 054	90
5		47	0	0 060	0 060	100
1	0 10	39	0	0 096	0 096	96
2		40	0	0 100	0 100	100
3		36	0	0 090	0 090	90
4		42	0	0 093	0 093	93
5		40	0	0 094	0 094	94
2	0 30	30	0	0 175	0 175	58
3		32	0 05	0 110	0 160	53
4		32	0 05	0 121	0 171	57
1	0 50	31	0	0 277	0 277	55
2		30	0 06	0 200	0 260	52
3		31	0	0 200	0 200	40
4		29	0 05	0 225	0 275	55
5		31	0	0 240	0 240	48
1	1 0	30	0 036	0 300	0 336	35
3		29	0 035	0 330	0 385	39
4		29	0 064	0 300	0 364	36
3	2 0	28	0 03	0 500	0 530	22
4		30	0 03	0 55	0 58	24
1	5 0	30	0 07	1 20	1 27	25
2		31	0 18	0 80	0 98	20
4		28	0 06	1 40	1 46	29
1	10 0	32	0 10	1 35	1 45	15
2		29	0 106	1 50	1 606	16
3		30	0 10	0 90	1 00	10
4		30	0 13	1 50	1 63	16

\* The original culture was divided into two portions which were hydrolyzed separately, and each value given was an average of values obtained from assaying several dilutions of each of the portions. All values fell within the range expected in microbiological assays,  $\pm 10$  per cent. All figures are given for the D isomer, assuming L biotin to be completely inactive.

As might be expected, however, the amount of biotin in the cells did not become constant beyond the point where enough biotin for maximum growth and acid production was present. Indeed, it seemed to be increasing as the biotin originally in the culture increased, even to the point where 100 times the amount necessary for maximum growth and acid production was furnished the organism. Thus, biotin, like thiamine, appears to be absorbed by the bacterial cells in huge quantities, the same is true for yeast (Chang and Peterson). The capacity of these cells for biotin is astonishingly high, more than 10 times what is needed for maximum function, as measured by cell growth and acid production, is present in cells furnished large excesses of biotin. This seems in contrast to cases of bacteria that synthesize their own biotin, in which it has been reported by Thompson (1942) that the largest amount of biotin is found released into the medium.

The results indicate that under the conditions of the present investigation *L. pentosus*, when grown in the presence of excess biotin, either metabolized some of the excess, or inactivated it in such a way that it was not detectable by the procedure used. Assays were also run using *S. cerevisiae* as the test organism, and the results were always found to be in close agreement with those using *L. pentosus*. This fact is some evidence that the biotin that could not be recovered was really metabolized, and not just slightly altered, for example to des-thiobiotin, since many reported biotin analogs are known to have activity for yeast.

Since there has been some controversy over the fate of oxybiotin in yeast (Rubin *et al*, 1945b, Hofmann, 1945, Hofmann and Winnick, 1945, Axelrod *et al*, 1946, 1947, Hofmann *et al*, 1947), it was of interest to study its fate in *L. pentosus* 124-2. Axelrod *et al* (1947) have proved that oxybiotin is assimilated as such by yeast, and is not converted into biotin. This also is the case with *L. pentosus*. Several levels of oxybiotin were used, and the results are given in table 4.

In no case was any biotin detected in the cells of *L. pentosus* grown in the presence of oxybiotin. A comparison of the columns for the total activity expressed as biotin, and the oxybiotin, indicates that no biotin was present in the cells and that the total biotin activity was due to oxybiotin. The differential assay for biotin showed no activity. Yeast assays were also run on these samples, and gave lower values than the *L. pentosus* assays, which would be expected if oxybiotin were the compound being measured.

It will be noted that the percentages of recoveries are somewhat lower for oxybiotin than for biotin (compare tables 3 and 4), but the differences are probably not significant. Axelrod *et al* (1947), in their studies on the metabolism of oxybiotin by yeast, obtained recoveries of over 70 per cent in all cases, which is high compared with those found here. It seems probable then that, although oxybiotin has not yet been found in natural materials, it is, from evidence so far available, able to elicit the same physiological response from some microorganisms as the naturally occurring biotin.

## DISCUSSION

From the data present here, the activities of biotin and oxybiotin for *L. pentosus* 124-2 appear to be identical. Their potency is the same on a weight basis within the experimental error of the method, they promote the same growth rate, and they are similarly metabolized by *L. pentosus*. This organism is evidently

TABLE 4  
*Metabolism of oxybiotin by Lactobacillus pentosus 124-2*

RUN NO	MILLIMICROGRAMS OXYBIOTIN PER ML ADDED TO MEDIUM BEFORE INOCULATION	EVELYN COLORIMETER READING 72 HR	MILLIMICROGRAMS BIOTIN OR OXYBIOTIN PER ML OF ORIGINAL CULTURE*							PERCENT AGE OF RECOVERY OF OXY BIOTIN
			Total activity expressed as biotin			Biotin	Oxybiotin			
			In medium	In cells	Total		In medium	In cells	Total	
2	0.04	50	0	0.040	0.040	0	0	0.042	0.042	105
3		48	0	0.050	0.050	0	0	0.044	0.044	110
4		48	0	0.045	0.045	0	0	0.042	0.042	105
3	0.06	46	0	0.048	0.048	0	0	0.046	0.046	77
4		45	0	0.046	0.046	0	0	0.045	0.045	75
3	0.10	41	0	0.068	0.068	0	0	0.060	0.060	60
4		41	0	0.070	0.070	0	0	0.066	0.066	66
2	1.0	30	0.036	0.220	0.256	0	0.035	0.250	0.285	29
3		31	0.040	0.300	0.340	0	0.035	0.260	0.286	29
1	5.0	30	0.054	1.20	1.254	0	0.044	1.10	1.144	23
2		33	0.06	0.87	0.93	0	0.056	0.82	0.876	18
3		32	0.06	0.80	0.86	0	0.070	0.76	0.83	17
4		30	0.06	0.82	0.88	0	0.060	0.83	0.89	18
2	10.0	29	0.06	0.80	0.86	0	0.07	0.74	0.81	8
3		30	0.044	1.25	1.294	0	0.036	1.18	1.216	12

\* The original culture was divided into two portions which were hydrolyzed separately, and each value given was an average of values obtained from assaying several dilutions of each of the portions. All values fell within the range expected in microbiological assays,  $\pm 10$  per cent. All figures are given for the D isomer, assuming L oxybiotin to be completely inactive.

not able to distinguish between the two compounds, each being assimilated as such. Since desthiobiotin has no activity for this organism, it is not able, under the circumstances of these experiments at least, to link the two carbons either with sulfur or with oxygen, a linkage seemingly necessary for activity. However, an organism that can use desthiobiotin, insofar as is now known, incorporates sulfur, not oxygen, into the molecule (Dittmer *et al.*, 1944, Leonian and Lilly, 1945, Tatum, 1945). It is conceivable that, if the required conditions were

presented the organism, oxygen might be used as the linking atom. Up to now, however, oxybiotin has not been found occurring naturally (Hofmann and Winnick, 1945, Luckey *et al*, 1946), but differential assays for oxybiotin on natural materials have been few.

In *L. pentosus* 124-2 the metabolism of oxybiotin is very similar to that of biotin. When only minimal amounts of biotin or oxybiotin are present, the organism seems to exercise a rigid economy, since nearly all can be recovered from the cells. When excess is present, it appears wasteful or even destructive of the biotin, as the lower recoveries indicate. McIlwain and Hughes (1944) found this true of hemolytic streptococci given an excess of calcium pantothenate. From the data of Axelrod *et al* (1947) yeast appears to be a more economical organism, since over 70 per cent of oxybiotin, even when excess was present originally, was recovered from the cells.

#### SUMMARY

DL-Oxybiotin had 50 per cent of the activity of biotin for *Lactobacillus pentosus* 124-2. Desthiobiotin had no activity, neither was it an inhibitor to biotin or oxybiotin.

The growth rate of *L. pentosus* 124-2 on graded amounts of biotin and oxybiotin has been determined. There was no significant difference in activity between the two compounds.

When minimal amounts of biotin or oxybiotin were furnished *L. pentosus* 124-2, all the activity of the added compound could be accounted for in the cells. Not until an excess of biotin or oxybiotin is present was any found left in the medium. As the amount of biotin or oxybiotin in the original culture increased, the percentage recovered decreased, and eventually reached only 10 to 15 per cent of that originally added to the medium.

Oxybiotin was assimilated as such by *L. pentosus* 124-2, and was not converted into biotin.

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# THE EFFECT OF RADIOACTIVE PHOSPHORUS UPON A SUSPENSION OF ESCHERICHIA COLI

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The effect of radioactive emanations upon microorganisms was apparently first studied by Pacinotti and Porcelli (1899), who exposed several organisms to uranium powder and stated that they were killed after 24 hours' exposure. Strebel (1900), Aschkinass and Caspari (1901), Crooks (1903), and Pfeiffer and Friedberger (1903) found the exposure of a variety of organisms to radium emanations to be toxic or to inhibit growth. Van Beuran and Zinsser (1903) and Prescott (1904) found no toxic or inhibiting effects of radium emanation on various species under the test conditions used. Chambers and Russ (1912) exposed aqueous suspensions of various organisms to radium emanation of 0.5 millicurie per ml and found sterility of the suspension to occur in 1 to 4 hours depending upon the species tested. This appears to be the first publication showing a quantitative estimate of the intensity of radioactivity used. Spencer (1934, 1935) introduced metal-covered radium therapy needles into freshly inoculated broth cultures of *Eberthella typhosa*, *Proteus* X19, and *Streptococcus pyogenes* and incubated the tubes. Growth was retarded for a short time but equaled the control tubes in 24 hours. When the tubes were held at 0 C, at which multiplication could not occur, the organisms appeared to die more rapidly in the irradiated tubes than in the control tubes. Lea, Haines, and Coulson (1936, 1937) and Lea, Haines, and Bretscher (1941) studied the effects of alpha, beta, and gamma rays on several species of organisms and found the organisms to be killed in an exponential manner.

In general, the results of these experiments with radioactive emanations indicate that some lethal effect upon microorganisms may be expected. The degree of the effect appears to depend upon the intensity and time of radiation, the type of the exposed organism, the conditions of exposure, and the nature of the radiation employed. It would be difficult, therefore, to predict at the present time whether any given radiation would have sufficient lethal effect to produce sterility in a food product.

In order to preserve food products in a sterile condition over a long period of time with safety to the consumer the application of a heating process is necessary. This is often accompanied by some degree of change in flavor, texture, and vitamin content of the product. For this reason many efforts are being made in the industry today to develop short-time high-temperature processes to minimize these effects. If such products could be sterilized by the incorporation of radioactive materials, the effects of heat could be eliminated. The development in recent years of methods for producing artificially radioactive isotopes in quantity has made available radioactive materials that might be used for

such purposes The experiment to be reported was therefore designed to test the sterilizing properties of radioactive phosphorus upon vegetative cells in a buffer solution containing no extraneous organic substances that could interfere with the maximum sterilizing effect of the radiation The premise upon which this experiment was designed was that if complete sterility of a suspension could

TABLE 1

*Arrangement of test to determine the survival of Escherichia coli suspended in phosphate buffer in the presence of radioactive phosphorus ( $P^{32}$ )*

	TUBE				
	A	B	C	D	E
Radioactivity in final cell suspension, microcurie/ml	0	1,000	500	100	50
ml radioactive phosphate 2,000 microcurie/ml	0	10	5	1	0.5
ml phosphate buffer 0.075 M	10	0	5	9	9.5
ml bacterial suspension 110,000,000 cells/ml in 0.075 M phosphate buffer	10	10	10	10	10

TABLE 2

*Survival of Escherichia coli suspended in phosphate buffer in the presence of various concentrations of radioactive phosphorus*

TIME OF EXPOSURE	INITIAL RADIOACTIVITY MICROCURIE PER ML									
	0		1 000		500		100		50	
	Surviving cells millions per ml (%)		Surviving cells millions per ml (%)		Surviving cells millions per ml (%)		Surviving cells millions per ml (%)		Surviving cells millions per ml (%)	
	ml	%	ml	%	ml	%	ml	%	ml	%
hr										
0	55.0	100.0	55.0	100.0	55.0	100.0	55.0	100.0	55.0	100.0
8	47.0	85.5	11.5	21.0	22.8	41.5	37.0	67.2	—	—
24	55.0	100.0	6.6	12.0	16.7	30.0	30.6	54.5	39.0	71.0
48	50.0	91.0	3.6	6.5	7.3	13.3	23.0	42.0	34.0	62.0
72	55.3	100.0	1.6	2.9	5.2	9.5	15.0	27.3	24.0	43.5
96	53.0	96.5	0.43	0.78	4.2	7.7	12.2	22.2	25.0	45.5
120	63.0	114.0	0.12	0.22	—	—	10.7	19.5	21.0	43.5
168	50.0	91.0	0.25	0.45	0.96	1.75	8.6	15.6	16.0	29.0
240	54.0	98.0	0.51	0.94	0.50	0.90	6.0	10.9	12.4	22.6
288	53.7	95.0	0.30	0.54	0.68	1.23	6.2	11.2	12.6	22.9

not be obtained under these most favorable conditions, the application of radioactive materials to the much more complicated conditions of a food product could not at present be considered practical

#### EXPERIMENTAL METHODS AND RESULTS

A solution of radioactive phosphorus ( $P^{32}$  emitting beta radiation) in the form of phosphoric acid was secured from the Radiation Laboratory of the Mas-

sachusetts Institute of Technology The solution was titrated electrometrically with NaOH to a pH of 6.95 and carefully evaporated. It was transferred to a 25-ml pyrex graduate cylinder and adjusted to a volume of 18.1 ml to give a solution 0.075 M in phosphate which would contain 2,000 microcuries of radioactivity per ml at the time the experiment was to be started. The solution was then sterilized at 15 pounds steam pressure for 15 minutes, and the slight loss in volume was restored aseptically with sterile water.

A suspension of *Escherichia coli* was prepared by washing 18-hour agar slants with sterile M/15 phosphate buffer, shaking with beads, and filtering through

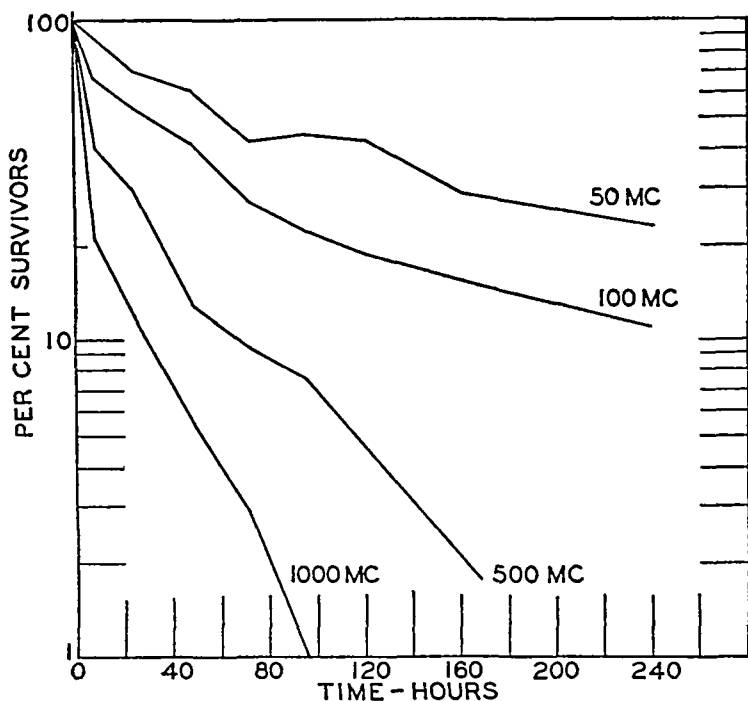


FIG. 1. THE SURVIVAL OF *ESCHERICHIA COLI* SUSPENDED IN PHOSPHATE BUFFER IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF RADIOACTIVE PHOSPHORUS ( $P^{32}$ )

cotton. The suspension was held at room temperature overnight while a plate count was made. The final stock suspension was made the following day by diluting with an equal volume of sterile water to give a suspension containing 110,000,000 cells per ml in 0.075 M phosphate buffer.

The experiment was started by combining radioactive phosphate solution, phosphate buffer, and bacterial suspension in the quantities shown in table 1, using 8-by-1-inch test tubes. After thorough agitation and mixing, 1-ml samples were removed from each tube for serial dilution and plate count. At least three dilutions were plated in triplicate for each sample. Further samples were taken at 8, 24, 48, 96, 120, 168, 240, and 288 hours for dilution and plating.

to determine the number of surviving cells. All plate counts were made after 48 hours' incubation at 98 F on Difco nutrient agar. The suspensions were held at 80 to 84 F during the experiment and were thoroughly shaken before each sampling.

The results of the experiment are shown in table 2 and illustrated in figure 1 in which the logarithms of the percentage of survivors are plotted against time of exposure.

#### DISCUSSION

It is evident from the data in table 2 that a very definite lethal effect is exerted on this organism by the presence of radioactive phosphorus in the solution. From figure 1 it is apparent that a uniform logarithmic rate of death did not prevail throughout the experiment, but that in each case the rate of death is markedly greater during the first 8-hour period than in the succeeding time intervals. Although no attempt has been made to draw smooth curves through

TABLE 3  
Calculated death rate for different time intervals for *Escherichia coli* in the presence of radioactive phosphorus ( $P^{32}$ )

TIME INTERVAL	INITIAL RADIOACTIVITY MICROCURIES PER ML			
	1 000	500	100	50
hours	K	K	K	K
0-8	0 085	0 047	0 022	—
8-24	0 015	0 008	0 0045	0 0037
24-240	0 016*	0 005	0 0037	0 0023

\* From 24 to 96 hours

the experimental points, it would appear that during the time interval from 8 hours to the end of the experiment an approximately logarithmic rate was maintained. These conclusions would seem to be supported by the calculations of death rate from the usual formula,

$$K = \frac{1}{t} (\log A - \log B)$$

where  $t$  = time interval in hours

$A$  = number of organisms present at beginning of the interval

$B$  = number of organisms present at the end of the interval

Table 3 shows the death rate calculated for different intervals and emphasizes the difference in death rate between the initial period and the subsequent period.

These results indicate that the suspension was not uniform in resistance but contained cells of differing susceptibility to the radiation such that the more sensitive cells were killed more rapidly at the beginning of the experiment. From the graph and from table 3, it will be noted that the death rate decreases

with decreasing intensity of radioactivity. Attempts to relate the logarithm of the number of survivors to radioactive dosage calculated as a function of intensity and time were not particularly successful in producing a curve uniformly expressing the results of the four intensity levels. Therefore these calculations are not presented. It is evident also from table 2 that complete sterilization was not obtained even with the highest concentration of radioactive phosphorus used. In the case of concentrations equivalent to 1,000 microcuries and 500 microcuries per ml it is very evident that a small fraction, approximately 1 per cent or less, of the cells are able to survive the radiation.

It seems apparent that at present successful application of radioactive materials to the sterilization of canned foodstuffs, at least, at the concentration levels tested is not indicated by this experiment. It also may be interesting to note that had sterilization been achieved the following would be the cost per no. 2 can for each of the concentrations tested: 1,000 microcuries \$3,660, 500 microcuries \$1,830, 100 microcuries \$366, and 50 microcuries \$183, based on the cost of radioactive phosphorus at the time the experiment was conducted.

#### SUMMARY

Radioactive phosphorus ( $P^{32}$ ) added to a suspension of *Escherichia coli* in phosphate buffer has a definitely lethal effect upon the cells that in a general way is related to the initial concentration. Complete sterilization of the suspension was not obtained in the highest concentration tested.

The survivor curve indicated a more rapid rate of killing during the first time interval and the existence of a small percentage of the cells that were more resistant and survived to the end of the experiment.

The present experiment does not suggest in any way the possibility of the application of radioactive materials to the sterilization of food products.

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## ANTIGENIC STUDIES OF A GROUP OF PARACOLON BACTERIA (BETHESDA GROUP)<sup>1</sup>

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In spite of continuous improvements in methods for the isolation of recognized pathogenic groups of *Enterobacteriaceae*, many outbreaks of gastrointestinal disease still occur in which only organisms of unknown significance are found. Often cases of diarrhea that are obviously of common origin yield only paracolon bacteria with identical or closely related biochemical characters. There is an increasing tendency to attribute etiological significance to these forms. The literature dealing with the role of paracolons in diarrhea has been reviewed by Neter and Clark (1944), Stuart and Van Stratum (1945), and Barnes and Cherry (1946).

One of the greatest difficulties in assessing the importance of paracolon bacteria in the causation of diarrheal disease is the lack of an exact method of identifying the cultures involved. In only a few instances has it been demonstrated that cultures from the same outbreak were serologically identical or even closely related. Rhodes (1942) found that several cultures isolated from one food-poisoning outbreak were identical. Stuart and Van Stratum (1945) found a high percentage of serologically identical coliform and paracolon strains in each of two institutional outbreaks of diarrhea in children. Barnes and Cherry (1946) noted that 28 strains isolated from an outbreak of gastroenteritis were serologically related, but the antigenic properties of the cultures were not studied intensively.

Although the Arizona group of paracolon bacteria has been classified and antigenic types have been established (Edwards, West, and Bruner, 1947), it composes only a very small fraction of the cultures usually classified as paracolon bacteria. No systematic work has been done on the antigenic analysis of other groups of these bacteria, although the work of Stuart, Wheeler, Rustigian, and Zimmerman (1943) demonstrated that a high degree of serologic relationship exists between biochemically similar strains. It therefore seemed worth while to study intensively the antigenic relations of a group of epidemiologically related strains in the hope of arriving at some conclusions regarding their significance. Also, such a study might serve as a basis for the classification of a further group of paracolons. Cultures previously described by Barnes and Cherry and similar cultures isolated from other small outbreaks were generously supplied by Comdr L A Barnes. Of the 32 cultures studied, 22 were

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isolated from 12 patients in one outbreak, 6 from 4 patients and 2 food handlers in a second outbreak, and 2 from patients in a third outbreak. The remaining 2 cultures were isolated from milk and from a person who developed diarrhea after drinking it. The cultures all belonged to the group classified by Stuart, Wheeler, Rustigian, and Zimmerman (1943) as paracolon intermediates and most closely resembled their type 14011. According to the scheme of Bormin, Stuart, and Wheeler (1944), they would be classed as *Paracolobacterium intermedium*. Since the majority of the cultures were isolated in Bethesda, Maryland, it is proposed to refer to this group of serologically related paracolons as the "Bethesda group."

#### BIOCHEMICAL PROPERTIES OF CULTURES

All the cultures were methyl-red-positive, Voges-Proskauer-negative types that failed to form indole but produced abundant  $H_2S$ . Barnes and Cherry (1946) found that they failed to grow in Koser's citrate medium, but all grew slowly on Simmons' citrate agar so that the medium became alkaline within 18 hours. All produced acid and gas from glucose, xylose, arabinose, rhamnose, maltose, trehalose, sorbitol, mannitol, and dulcitol within 24 hours. Lactose was acidified in 3 to 6 days, and slight gas production was eventually noted in all but 2 cultures. Eleven cultures fermented sucrose within 24 hours, but the remainder did not attack the sugar after 3 serial transfers at intervals of 30 days. Sucrose fermentation was confined to one serologic type, although not all cultures of that type fermented sucrose. Sucrose-positive and sucrose-negative cultures that were serologically identical were isolated from the same individual. Salicin was fermented by half of the cultures after 10 to 25 days' incubation. There was no correlation between salicin fermentation and serologic type, and salicin-positive and salicin-negative strains were isolated from the same individual. All but 3 of the cultures fermented cellobiose after 3 to 10 days. Inositol was not fermented. Gelatin was not liquefied after 90 days' incubation. Litmus milk was slowly acidified but was not coagulated during 60 days' incubation.

#### SEROLOGIC STUDIES

Any study of the antigens of paracolon bacteria must be approached with some caution since it has been shown by Kauffmann (1943, 1944) and by Knipschuldt (1945) that coliform bacteria contain heat-labile and heat-stable somatic antigens that inhibit O agglutination. Therefore, it is necessary to ascertain whether such antigens are present in cultures being studied. O serums were prepared from broth cultures that had been heated at 121 C for 2.5 hours. The serums were then used in agglutination tests in which living broth cultures and broth cultures heated to 100 C served as antigens. Tests were incubated at 50 C for 1 hour and read after standing overnight. In the strains under consideration it was found that living cultures and heated cultures were agglutinated equally well and in high dilution by the O serums, a fact which indicated that they did not contain L or A (K) antigens. Though tube agglutina-

tion was used throughout the work, slide agglutination tests with properly diluted serums gave comparable results

The H antisera were prepared from formalinized broth cultures of strains that had been passed repeatedly through semisolid medium to insure optimum development of H antigens. Similar cultures were used as antigens in the H agglutination tests, which were read after incubation for 1 hour at 50 C.

By agglutination and absorption the organisms were divisible into four O groups, the reactions of which are given in table 1. Fifteen cultures fell into group 1, 7 into group 2, 10 into group 3, and 1 into group 4. The O antigens of all group 1 cultures were identical, as were also those of group 2. In group 3 minor differences were found among the cultures and their relationships were complicated. Reciprocal absorptions revealed minor differences that were not clear cut. For the present it seems best to place these strains in one O group.

The O antigens of the cultures were tested with serums for Kauffmann's coli O groups 1 to 11 inclusive, 18, and 21 to 25 inclusive. These serums were ob-

TABLE 1  
*Somatic agglutination and O factors*

ANTIGENS	SERUMS						
	Na 1A (1)		Na 4 (2)		Na 11 (3)		Na 19 (4)
	Unab-sorbed	Absorbed by Na 4 (2)	Unab-sorbed	Absorbed by Na 1A (1)	Unab-sorbed	Absorbed by Na 1A (1)	Unab-sorbed
Na 1A (1)	12,800	6,400	3,200	0	6,400	0	0
Na 4 (2)	3,200	0	12,800	12,800	1,600	0	0
Na 11 (3)	0	0	0	0	12,800	3,200	0
Na 19 (4)	0	0	0	0	0	0	6,400

0 indicates no agglutination 1 100

Figures in parentheses indicate O factors

tained through the kindness of Dr. Kauffmann. The paracolons of O groups 1 and 3 were agglutinated to 5 to 10 per cent of the titer of coli O group 9 serum. No other reactions were noted. Coli 9 was not agglutinated by the paracolon serums. The cultures included in this study were not agglutinated by any *Salmonella* O serums. Other strains of the Bethesda group that have been studied but that are not included here have somatic relationships to *Salmonella onderstepoort*.

Five groups of H antigens were established by agglutination and absorption tests. These are set forth in table 2. The 15 cultures included in H group 1 were identical, as were the 3 cultures of H group 2, and the 5 strains of H group 4. The 6 strains included in H group 3 were very closely related but minor differences were found in absorption tests. The 2 cultures of H group 5 cross-agglutinated in high dilution, but even in the agglutination tests it was apparent that differences existed between them. These differences were clearly revealed by absorption tests, but until more is known about the antigens of the group it seems best not to separate them.

When the O and H groupings of the 32 cultures were combined, 8 types were established. These types were designated simply by the numbers applied to the O and H groups to which they belonged. Thus a member of O group 2 that had group 3 H antigens was represented by the formula Be 2 3. The letters preced-

TABLE 2  
*Flagellar agglutination and H factors*

ANTIGENS	SERUMS								
	Na 1A (1)	Na 2C (2)		Na 23 (3)		Na 4 (1)		Na 11 (5)	
	Unab-sorbed	Unab-sorbed	Absorbed by Na 23 (3)	Unab-sorbed	Absorbed by Na 2C (2)	Unab-sorbed	Absorbed by Na 11 (5)	Unab-sorbed	Absorbed by Na 11 (5) + Na 4 (4)
Na 1A <sub>3</sub> (1)	12,800	0	0	0	0	0	0	0	0
Na 2C <sub>3</sub> (2)	0	12,800	12,800	800	0	0	0	0	0
Na 23 (3)	0	400	0	12,800	6,400	0	0	200	0
Na 4 (4)	0	0	0	0	0	12,800	6,400	400	0
Na 11 (5)	0	0	0	0	0	800	0	6,400	1,600

0 indicates no agglutination at 1:100

Figures in parentheses indicate H factors

TABLE 3  
*Distribution of serologic types*

OUTBREAK	TYPE	NO CULTURES	NO PERSONS	DIARRHEA
1	1 1	15	9	+
	2 3	1	1*	+
	3 2-3	4	1	+
	3 3	1	1	+
	3 5	1	1	+
2	2 4	3	3†	+
	3 3	1	1	-
	4 3	1	1	-
3	3 3	1	1	+
	3 5	1	1	+
4	2 4	2	1‡	+

\* Type 1 1 also recovered from this patient

† Nonmotile culture of O group 2 recovered from fourth patient

‡ One culture from milk consumed by patient

ing the formula indicate that the culture belonged to the Bethesda group. The distribution of the types is given in table 3. Fifteen cultures of Be 1 1 were recovered from 9 of 12 patients in outbreak 1. One of these patients also yielded a Be 2 3 culture. Four cultures of Be 3 2-3 were recovered from one patient. These cultures are discussed later. Types Be 3 3 and Be 3 5 were each recovered from one patient. In the second outbreak 3 of 4 persons affected yielded

type Be 2 4, but a nonmotile strain of O group 2 was recovered from the fourth. Two food handlers who were not ill yielded types Be 3 3 and Be 4 3, respectively. The third outbreak was composed of only 2 cases of diarrhea thought to be of common origin. One patient yielded type Be 3 3, the other type Be 3 5. The fourth outbreak was represented by only 2 cultures, one from milk that obviously was unfit for consumption and the second from a person who developed diarrhea after drinking the milk. The cultures were identical and belonged to type Be 2 4.

#### VARIABILITY IN CULTURES

The reactions of the O groups given in table 1 suggest that form variation similar to that described in the *Salmonella* group by Kauffmann (1940) exists in these strains. This possibility has not been investigated. Certain observations were made on variation in the H antigens, in which the O antigens were unaffected. One patient in outbreak 1 yielded 4 cultures of Be 3 2-3. Actually, three of the cultures were isolated as Be 3 2 and one as Be 3 3. Since the O antigens of the cultures were identical, this strongly suggested that H antigens 2 and 3 had a common origin, although, as shown in table 2, there was very little relationship between them. By growing the three Be 3 2 cultures in semisolid agar that contained appropriately absorbed H group 2 serum, it was possible to change them to Be 3 3. Likewise, it was possible to change the Be 3 3 culture recovered from this patient to Be 3 2 by cultivation of the culture in semisolid medium containing absorbed serum of H group 3. That the H antigens of the cultures were actually reversed was shown by reciprocal absorption tests. Thus, these cultures must be considered as one type that has two H phases, and the formula must be written 3 2-3. This observation is very similar to that of Kristensen and Bojlen (1936), who isolated two cultures with the formula VI,VII 1,5 and one culture with formula VI,VII c from the stools of a patient infected with *Salmonella cholerae-suis* var *kunzendorf*.

Within type Be 3 5 are two cultures, Na 11 and Na 22. Both the O and H antigens of these cultures are closely related, but neither the O nor H components of the two are identical. It was found that Na 11 was related to Md 2, a culture received from Mr. A. A. Hajna. When a single colony culture of Na 11 was plated, two distinct forms were isolated, the reactions of which are given in table 4. These forms, which are called phase 1 and phase 2, were both agglutinated in high dilution by serum derived from the whole culture. Phase 1 (H antigen 6) was agglutinated in high dilution by Md 2 serum but was unaffected by Na 22 serum. With phase 2 these reactions were reversed. Colonies of mixed phase were found that were agglutinated by both serums. Upon plating these mixed colonies, mixed colonies and colonies of phase 2 appeared. When phase 2 colonies were placed in semisolid medium that contained suitably absorbed Na 22 serum, they spread through the medium and phase 1 was isolated from the spreading growth. Phase 1 has not been similarly reversed, nor has either phase been found to revert naturally to the other.

As originally received, Na 22 was agglutinated actively by Na 11 serum, and the two cultures were included in the same H group. When this culture was

placed in semisolid medium that contained suitably absorbed Na 11 serum, it spread through the medium, and from the spreading growth a form was isolated that was no longer agglutinated by Na 11 serum and reacted only slightly with serum derived from the parent strain. The changed culture was agglutinated to the titer of serum derived from Md 10, another strain received from Mr Hajna. This component was called phase 1 and designated as H antigen 7.

TABLE 4  
*Variation in H antigens*

ANTIGENS	H FACTORS	SERUMS			
		Na 11	Na 22	Md 2	Md 10
		5 6	5	6	7
Na 11, whole culture	5, 6	12,800	6,400	3,200	0
Na 11, phase 1	6	12,800	0	6,400	0
Na 11, phase 2	5	12,800	12,800	0	0
Na 22, original	5	3,200	12,800	0	200
Na 22, induced	7	0	200	0	6,400
Md 2	6	800	0	12,800	0
Md 10	7	0	0	0	6,400

0 indicates no agglutination at 1:100

TABLE 5  
*Antigenic table*

ANTIGENIC SYMBOLS			TYPE STRAINS
O antigens	H antigens		
	Phase 1	Phase 2	
1	1		Na 1a
2	4	3	Na 23 Na 4
3	2	3	Na 2C Na 12, Na 20, Na 30
	6	5	Na 11
	7	5	Na 22
4		3	Na 19

The only variation noted in Na 22 was that which occurred under the influence of serum.

#### ANTIGENIC TYPES

The antigenic types established are given in table 5. The 4 O groups were divided into 8 serologic types. Type 3:3 was not merged with type 3:2-3 nor designated as a subtype of the latter since the cultures were not identical with phase 2 of 3:2-3 nor with each other. It was stated above that O group 3

and H group 3 each contained closely related but distinct antigens. Thus cultures Na 12, Na 20, and Na 30 possess slightly different O and H antigens. At present these types are placed under the same antigenic formula because the specific factors that distinguish them are rather weak. Later it may be found advisable to distinguish between them. As yet these strains have not been found diphasic. It is possible that they later may be distinguished by the isolation of other phases.

#### DISCUSSION

The question of the pathogenicity of the Bethesda group is not definitely answered by this study, but certain suggestive results were obtained. Although the paracolons isolated from outbreak 1 were divisible into 5 types, one type (Be 1 1) was predominant and was present in at least 9 of 12 patients from which cultures were available. The presence of this type in 75 per cent of the individuals suggests that it was responsible for the epidemic.

In the second outbreak 3 of the 4 persons affected yielded identical cultures, but from the fourth patient was isolated a nonmotile culture of the same O group. Asymptomatic food handlers connected with this outbreak yielded distinctly different types. It seems highly probable that type Be 2 4 was responsible for this infection.

The third outbreak consisted of only 2 cases of diarrhea thought to be of common origin. These yielded types of the same O group that had distinct H antigens. One of these cultures was Na 11, mentioned above. Since so little is known of variation in these organisms, it is impossible to say whether the 2 cultures originated from the same type. The fourth outbreak was represented only by 2 identical cultures, one from obviously spoiled milk and the other from the stools of a patient who became ill after drinking it.

Although the results suggest that these paracolon bacteria may incite outbreaks of disease, it was found that many cultures of the Bethesda group occurred in individuals with no history of diarrhea. This group is widely distributed, and the writers are in possession of a large number of cultures from both normal persons and individuals affected with diarrhea. If the organisms are pathogenic, it must be recognized that the carrier-case ratio is rather high, apparently much higher than in the *Salmonella* and in the *Shigella* types of recognized pathogenicity.

In the present state of knowledge it is difficult to establish the limits of the Bethesda group. As in other enteric bacteria it undoubtedly will be found to merge with other groups. Nevertheless, until more is known of the serology of the *Enterobacteriaceae* it seems worth while to set it apart for the moment so that it may be studied and correlated with other groups. Such a procedure has yielded excellent results with the true *Escherichia coli* types and with the Arizona paracolons. Therefore, the Bethesda group is tentatively described as follows:

Slow lactose fermenting cultures that are methyl-red-positive, Voges-Proskauer-negative, indole-negative, H<sub>2</sub>S-positive, D-tartrate positive and Simmons' citrate-positive. Urea utilization is negative when tested by the method of Rustigian and

Stuart (1941), but most cultures give positive tests by the method of Christensen (1946). Dulcitol usually is fermented promptly, although dulcitol negative strains occur. Sucrose generally is not fermented, although occasional cultures may ferment sucrose.

Undoubtedly, cultures with other biochemical characters will be found to be serologically related to this group. However, it is within this biochemical group that serologic relationships most frequently are found. It is known that the antigens characterized here are only a few of many that occur within the group and that the establishment of the few serologic types delineated here constitutes only a very meager beginning in its classification. However, if these cultures that prove so troublesome to the worker in enteric bacteriology are to be understood a start toward their classification must be made.

The few instances of serologic variation noted above indicate that the classification of the group will not be easily accomplished. For lack of landmarks the work proceeded slowly, and it was necessary to support each step by reciprocal absorption tests. As the group is better understood its classification should proceed much more rapidly.

#### SUMMARY

Thirty-two paracolon cultures isolated from 4 distinct outbreaks of diarrhea were studied. The organisms formed a rather uniform biochemical group. They were divided into 8 serologic types through the examination of their O and H antigens. In general, the types established by serologic examination agreed fairly well with the epidemiologic data. The H antigens of some cultures were variable. The cultures studied belonged to a group of paracolon bacteria that were designated as the Bethesda group.

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# THE IDENTIFICATION OF ANTIBIOTICS BY MEANS OF RESISTANT STRAINS OF BACTERIA

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In the search for new antibiotic substances, a general method that would permit the prompt recognition of new types of antibiotics (i.e., antibiotics with different mechanisms of action) and at the same time reduce the risk of undertaking costly investigation of an unidentified substance which may not be new would clearly be advantageous. A means for achieving this purpose was suggested some time ago (Stansly, 1946). It was proposed that strains of bacteria specifically resistant to the various known antibiotics should be prepared from one or more designated parent strains, and the resistant strains then filed with an agency, such as the American Type Culture Collection, in order that they might be readily accessible and available upon request. Strains of bacteria specifically resistant to the known antibiotics would enable investigators to determine in a systematic fashion very early in an investigation whether an unidentified antibiotic was different from or similar to any one of the known substances. This will be elaborated upon further and examples illustrating the method described.

For such a scheme to be of maximum effectiveness, none of the known antibiotics should be neglected. The preparation of resistant strains and, when necessary, of the antibiotic substances required to accomplish this then becomes a task of some magnitude. It is apparent, therefore, that the success of the proposed plan is dependent upon the willingness of interested laboratories to collaborate actively in undertaking a project of this sort. Future responsibility for preparing resistant strains to new antibiotics would normally be expected to fall upon the investigators first describing such new antibiotics. The investigators, while unable, or perhaps unwilling, to distribute samples of the antibiotic or the antibiotic-producing organism, should have no hesitancy in making available strains of bacteria specifically resistant to the new antibiotic.

*Use of resistant strains of bacteria.* The following model will illustrate the application of the proposed plan. Suppose  $P, R_1, R_2, R_3, R_4, \dots, R_n$  to be the parent and strains derived therefrom that are resistant to the known antibiotics  $A_1, A_2, A_3, A_4, \dots, A_n$ . Let the inhibition ratio,  $R/P$ , determined under standard conditions for each of the antibiotics be 256, 64, 1024, 512, 128, respectively. Under similar conditions, an unidentified antibiotic "X" is tested against the parent and each of the resistant strains (it is immaterial, of course, whether X is tested in terms of mg per cent, dilution units, etc.) and the resulting ratios  $R_1/P, R_2/P, R_3/P, R_4/P, \dots, R_n/P$  found to be 1, 1, 1, 512,

A comparison of the ratio for X and of those for the known antibiotics indicates that it is equivalent only to that of A<sub>4</sub>, thus providing *presumptive* evidence for the identity of X and A<sub>4</sub>. On the other hand, the divergence from equivalence (which must, of course, be outside the experimental error) in the cases of A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>, A<sub>n</sub> may be considered fairly *conclusive* evidence that X is not A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>, A<sub>n</sub>. Thus, a *difference* in inhibition ratios is more significant than a similarity. X, in other words, may be identical with A<sub>4</sub>, but further investigation would be required if it is desired to establish this point beyond doubt. This limitation of the application of resistant strains for the purpose of "identification" has been discussed by Eisman, Marsh, and Mayer (1946) and Kelner and Morton (1947). The former authors note, however, that "it is likely that antibiotics of *diverse* [italics mine] chemical structure can be differentiated by this method." This is precisely the purpose of the proposal presented here, that is, to provide a means for promptly detecting antibiotics with different mechanisms of action and presumably, therefore, of significantly different chemical structures.

Conceivably, mixtures of antibiotics, such as might occur in crude culture filtrates or in crude concentrates, may give less than the clear-cut results shown above. To illustrate, suppose a crude culture filtrate "X" to consist of 1 part of streptothricin and 31 parts of streptomycin. An analysis with streptomycin- and streptothricin-resistant strains having standard inhibition ratios of 1,024 and 128, respectively, might give the theoretical results shown in table 1.

It might be concluded on the basis of the hypothetical results shown in table 1 that X is not streptothricin and probably not streptomycin (though possibly related to it in mechanism of action). In a sense, this is true. X is neither streptothricin nor streptomycin but a mixture of the two in the proportion of 1 to 31. It would appear, therefore, that unidentified substances giving inhibition ratios with certain resistant strains significantly different from unity yet not equivalent to any of the "standard" ratios might be suspected of consisting of a mixture of antibiotics, one or more of which is known. Furthermore, the magnitude of the ratios observed with the unidentified substance bears a rough relationship to the proportions of the active substances in the mixture.

Unidentified substances which satisfy the criteria given above would perhaps be considered worthy of further investigation on the premise either that one or more components of the supposed mixture may be unknown or that the unidentified agent may be a single substance which has a similar though not identical mechanism of action to one of the known antibiotics. Whether or not further investigation is indicated depends upon the aims of the investigation.

In our limited experience, the use of specifically resistant bacteria for the purpose of identification has given results not far different from the hypothetical model discussed earlier. A few examples from our laboratory will serve as illustrations. Others may be found in the literature (Eisman *et al.*, 1946; Kelner and Morton, 1947; Stanley and Mills, 1946; Truaxell, Fulton, and Grant, 1947; Waxman, Reilly, and Schatz, 1945).

The examples to be described are not model experiments designed for purposes

of illustration but were planned to meet problems in the laboratory as they arose. Whenever possible, specifically resistant strains were tested with the known and unknown antibiotics simultaneously. It should be stressed, however, that the plan proposed does not require the various known antibiotics (which are generally unavailable) for the performance of the identification tests. All that is required are the parent and resistant strains, together with their inhibition ratios determined under standard conditions.

*Examples illustrating the practical application of resistant strains of bacteria*<sup>1</sup>

(1) An antibiotic-producing actinomycete (A9) was isolated from soil. The

TABLE 1

*Hypothetical effect of a mixture (X) of streptomycin and streptothricin (31:1) on resistant strains of bacteria*

ANTIBIOTIC	INHIBITION END POINT WITH STRAIN*		
	P	R <sub>SM</sub>	R <sub>STH</sub>
X	1	32	1
Streptomycin	1	1,024	
Streptothricin	1		128

\* P = parent, R<sub>SM</sub> = streptomycin-resistant strain derived from P, and R<sub>STH</sub> = streptothricin-resistant strain derived from P. Inhibition end points are expressed in arbitrary units.

TABLE 2

*Presumptive identity of A9 with streptothricin*

ANTIBIOTIC	INHIBITION END POINT WITH STRAIN*				INHIBITION RATIO	
	P <sub>1</sub>	R <sub>1SM</sub>	P <sub>2</sub>	R <sub>2A9</sub>	R <sub>1SM</sub> /P <sub>1</sub>	R <sub>2A9</sub> /P <sub>2</sub>
A9	1/16	1/4	1/256	1/2	4	128
Streptomycin	1	1,024			1,024	
Streptothricin			1/2	64		128

\* P<sub>1</sub> and R<sub>1SM</sub> = parent and streptomycin-resistant *Escherichia coli*; P<sub>2</sub> and R<sub>2A9</sub> = parent and A9-resistant *Bacillus subtilis*. Inhibition end points are expressed as a dilution for A9 (a broth filtrate) and as mg per cent for streptomycin and streptothricin.

question of the identity of the substance produced by this organism with streptomycin or streptothricin arose. The response of certain parent and resistant strains of bacteria was determined with the results shown in table 2.

It is apparent from the data shown in table 2 that A9 could not be (at least, solely) streptomycin. They suggest, on the other hand, that A9 contained predominantly streptothricin. In view of this, we elected not to investigate the antibiotic A9 further.

<sup>1</sup> We are indebted to Dr. H. J. White and Mrs. A. H. Clapp of these laboratories for all but one of the resistant strains used in these examples and for the data on the comparison of polymyxin and streptomycin.

(2) It is common experience that antibiotic activity may be demonstrated more readily in the region surrounding a colony of the antibiotic-producing organism on an agar plate than it can in broth. A means of gaining a clue as to the nature of an unidentified substance before much effort is expended obtaining active metabolite solution is obviously advantageous. An illustration with A9 will demonstrate the point.

TABLE 3  
*Inhibition zones produced by actinomycete A9*

ORGANISM	STRAIN*	INHIBITION ZONE (MM)
<i>E. typhosa</i>	P	13.5
	R <sub>SM</sub>	14.0
<i>P. aeruginosa</i>	P	8.5
	R <sub>STH</sub>	0

\* P = parent strain

R<sub>SM</sub> = streptomycin-resistant *Eberthella typhosa*

R<sub>STH</sub> = streptothricin resistant *Pseudomonas aeruginosa*

TABLE 4  
*Nonidentity of polymyxin and streptomycin*

ANTIBIOTIC	INHIBITION END POINT	(MG %) WITH STRAIN*	INHIBITION RATIO
	P	R <sub>SM</sub>	R <sub>SM</sub> /P
Polymyxin	2	2	1
Streptomycin	1	>512	>512

\* P = parent *Escherichia coli* R<sub>SM</sub> = streptomycin resistant strain derived from P

TABLE 5  
*Presumptive identity of B71 with polymyxin*

ANTIBIOTIC	INHIBITION END POINT U/ml WITH STRAIN*				INHIBITION RATIO	
	P <sub>1</sub>	R <sub>1PM</sub>	P <sub>2</sub>	R <sub>2PM</sub>	R <sub>1PM</sub> /P	P <sub>2PM</sub> /P
Polymyxin	12.5	1,600	12.5	200	128	16
B71	25.0	1,600	6.25	200	64	32

\* P<sub>1</sub> and R<sub>1PM</sub> = parent and polymyxin resistant *Pseudomonas aeruginosa*

P<sub>2</sub> and R<sub>2PM</sub> = parent and polymyxin resistant *Klebsiella pneumoniae*

The organism producing A9 was streaked across an agar plate. Following growth, the plate was cross-streaked in the usual fashion with a streptomycin-resistant strain of *Eberthella typhosa* and its parent as well as with a streptothricin-resistant strain of *Pseudomonas aeruginosa* and its parent. The zones of inhibition were measured after incubation overnight. The results are shown in table 3.

It was apparent from the results of this simple procedure (table 3) that A9 was not streptomycin but was either identical with or closely related to streptothricin

(3) In the early stages of investigation, the antibiotic polymyxin (Stansly, Shepherd, and White, 1947) appeared to resemble streptomycin and streptothricin in certain of its properties. It was felt desirable to determine whether polymyxin was identical with either. One of the ways this was tested was by the use of resistant strains, as shown in table 4

It was apparent from the results shown in table 4 that polymyxin was not identical with streptomycin. Similar results were obtained with a streptothricin-resistant strain and its parent. The lack of identity of polymyxin with both streptomycin and streptothricin was confirmed in other ways

(4) A laboratory<sup>2</sup> had an antibiotic ("B71") whose identity with polymyxin was in question. Upon analysis with polymyxin-resistant bacteria, the results shown in table 5 were secured

It was apparent from these results that polymyxin and B71 were either identical or closely related. Results from other biological tests, as well as from chromatographic analysis, degradation products, and infrared spectra, confirmed the probable identity of the two substances

#### DISCUSSION AND COMMENT

A major point of this communication is that, for the reasons pointed out, the project proposed must be undertaken as a joint effort of the various laboratories engaged in antibiotic investigation

Emphasis in this proposal is placed upon resistant strains derived from one or more designated parent strains of bacteria. The plan could, however, also include pairs of unrelated organisms, one of the pair being sensitive, the other naturally resistant to the known antibiotic. Pairs of this type have, of course, been valuable in the past and would be useful expedients should it prove impracticable to secure resistant "mutants" to certain antibiotics

With respect to resistant mutants, spontaneous decrease or loss of resistance has been envisaged by some as a possible obstacle to the successful operation of the proposed plan. The incidence of instability of resistant strains and hence the seriousness of the problem is, however, more or less unknown. Furthermore, it is unlikely to be settled for some time to come. It would seem inadvisable to wait for this event to occur before taking steps to improve an increasingly difficult situation, namely, that of determining the possible identity of an antibiotic with one already reported. The plan for the identification or differentiation of antibiotics may be looked upon as an experiment with much to be gained should it prove capable of giving useful information to investigators in the field. The fact that not all the answers are at hand to meet the problems that are envisaged need not be a deterrent to instituting an adequate trial to determine the utility of the project. One beneficial result would be that knowledge of the

<sup>2</sup> Lederle Laboratories Division, American Cyanamid Company

behavior of resistant strains would of necessity accumulate. The scheme could then be modified accordingly.

From a practical standpoint several expedients are available either to minimize complications caused by the loss of resistance or to minimize the loss of resistance itself. The former problem can be met by periodic testing of resistant strains by the issuing agency or by authorized laboratories. The latter problem can be met in either of two ways: (1) by preparing a massive culture of the resistant strain, which is then lyophilized and distributed in the dry state, or (2) by culturing, when feasible, resistant strains in antibiotic-containing media.

#### SUMMARY

A plan is proposed offering a systematic approach to the problem of early identification of unknown antibiotics and of prompt recognition of new types of antibiotics by means of resistant strains of bacteria. Collaboration on the part of interested laboratories would be essential for the initiation of the plan.

The various known antibiotics (which are generally unavailable) are not required for the performance of the identification tests. All that is required are the various antibiotic-resistant strains together with their "inhibition ratios" determined under standard conditions.

A model illustrating the application of resistant strains to the problem presented is given together with a number of examples illustrating the utility of the method.

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# CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

## VI THE INFLUENCE OF COBALT ON THE OPTIMAL BACTERIOSTATIC CONCENTRATION OF PENICILLIN<sup>1</sup>

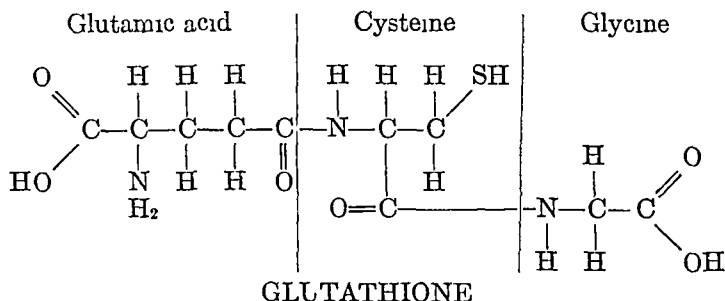
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It has been shown that the antibacterial action of penicillin *in vitro* and *in vivo* may be markedly enhanced by the presence of appropriately low concentrations of cobalt that do not themselves inhibit proliferation of the test organisms (Pratt and Dufrenoy, 1947*b*, Pratt, Dufrenoy, and Strait, 1948, Strait, Dufrenoy, and Pratt, 1948). This paper presents further evidence of the enhancing action of cobalt on penicillin activity and offers an explanation of the phenomenon. It also attempts to define the factors that determine the threshold concentration below which penicillin fails to check and above which it checks the normal tendency of the test organism to proliferate. The question of an "optimal" concentration of penicillin, i.e., whether concentrations above the minimal effective level retard the action of penicillin or not, is also discussed.

Growth of *Staphylococcus aureus* is dependent on an external source of —SH groups, such as presumably may be converted to cysteine (Fildes and Richardson, 1937) and on an external source of glutamic acid (Gale and Taylor, 1946, 1947). The observations of Gale and coworkers (1946, 1947) as well as results of our own cytochemical studies (Dufrenoy and Pratt, 1947*a,b*, Pratt and Dufrenoy, 1947*a,b*, 1948) suggest that glutathione (γ-glutamyl-cysteinyl-glycine), resulting from the linkage of glutamic acid to glycine via cysteine, as follows,

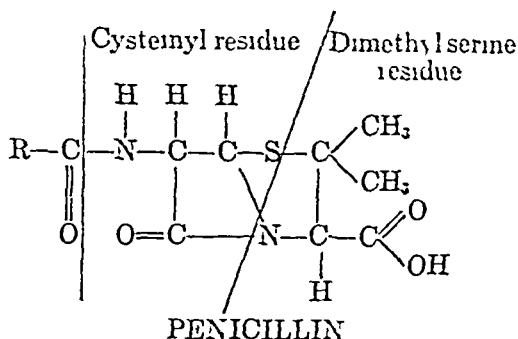


may be involved in the mechanism of bacteriostasis of gram-positive organisms by penicillins, since the various penicillins may be written as stereochemical

<sup>1</sup> This work was supported in part by a generous research grant from the Cutter Laboratories, Berkeley, California

<sup>2</sup> With the capable and gracious laboratory assistance of Virginia Lamb

analogues of glutathione with glycine replaced by dimethyl serine, and the glutamyl fraction replaced by an R residue as follows,



The similarity in structure of glutathione and penicillin has been commented upon in several reports, including those of Fischer (1947) and Pratt and Dufrenoy (1948)

Gale and coworkers (1946, 1947) have shown that organisms that depend for their survival on absorption of glutamic acid from the substrate are penicillin sensitive. Our own studies of staining reactions on assay plates seem to point directly to the involvement of glutathione in the action of penicillin. When assay plates are treated with the Prussian blue reagent for the detection of  $-SH$  groups, the ring of enhanced growth that circumscribes each zone of inhibition stains an intense blue (Dufrenoy and Pratt, 1947a, Pratt and Dufrenoy, 1947b), the positive reaction is not obtained, however, if the  $-SH$  groups have been blocked by bromacetate before addition of the ferricyanide ferric sulfate reagent (Dufrenoy and Pratt, 1948). These observations suggest that the over-all effect of penicillin may be traceable ultimately to dehydrogenation of reduced glutathione to oxidized glutathione, resulting largely from the increased rate of metabolism that is induced by appropriately low concentrations of penicillin. The most efficient bacteriostatic or bactericidal concentration of penicillin may be visualized as one that promotes dehydrogenation faster than rehydrogenation can restore the  $-SH$  groups, thus shifting the aerobic respiratory system out of balance and depriving the microorganisms of the respiratory energy required to provide for absorption of essential metabolites from the substrate.

If these assumptions are correct, it follows that penicillin will be most effective at a concentration such that it accelerates the transfer of hydrogen from  $-SH$  groups without impeding the activity of hydrogen acceptors. Therefore, for each set of experiments *in vitro* or *in vivo* there would be expected a threshold value, below which the antibiotic speeds up metabolic processes without irreversibly shifting them out of balance, and above which it increases dehydrogenation beyond the rate compatible with maintenance of the proper redox systems and thereby causes irreparable damage to the cells. According to this hypothesis penicillin at an appropriate subthreshold concentration could be expected to act as a "growth factor," and it is noteworthy that such a fact

has been reported repeatedly (see review by Pratt and Dufrenoy, 1948). Concentrations greatly in excess of the threshold would be expected to impair the effectiveness of H acceptors and so partially to protect H donors against rapid, irreversible dehydrogenation. Therefore, it would be predicted that the most efficient and rapid bacteriostatic effect would follow use of the minimal concentration capable of inducing irreversible injury to the cells.

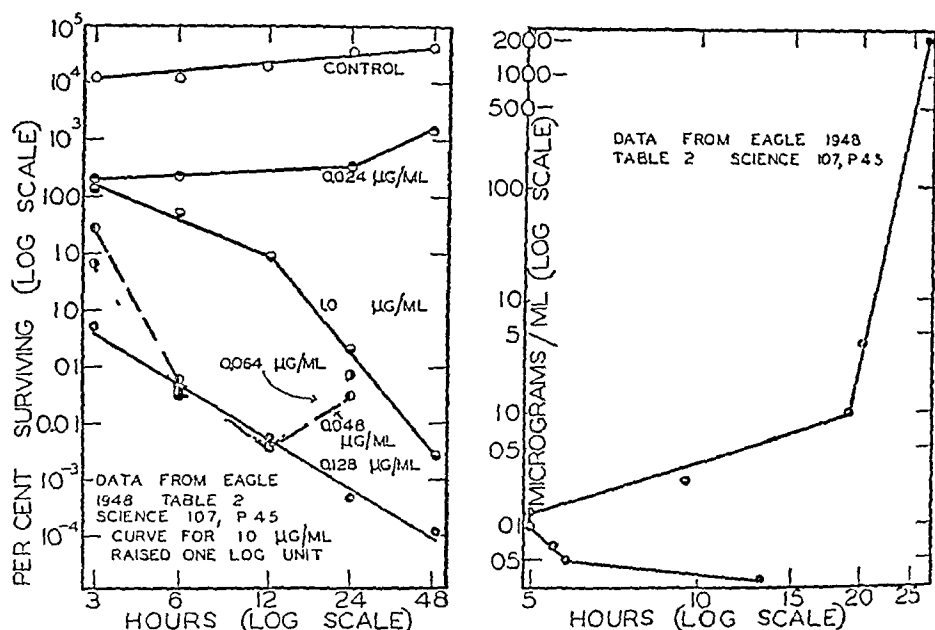
Experimental evidence to support this concept is provided by the data of Abraham and Duthie (1946) and Eagle (1948), and by the results of our own researches. Abraham and Duthie observed that "after 22 hours, more than twice as many bacteria were visible in the presence of 0.04 units per c. cm. as in the presence of 0.03 units per c. cm." Eagle showed that for the organisms he studied there are three critical concentrations of penicillin, including an "optimal" concentration at which the organisms are killed at a maximal rate. In figure 1, data for *S. aureus*, taken from Eagle's table 2, are plotted on a log-log scale, showing the percentage of organisms surviving (referred to original inoculum as 100) as ordinates and the hours of exposure to penicillin as abscissae. In the presence of 0.024  $\mu$ g penicillin per ml, the curve is shifted downward on the ordinate. This represents a concentration that, in Eagle's words "serves only to reduce the rate of multiplication." At higher concentrations, 0.048 and 0.064  $\mu$ g per ml, organisms die faster than they multiply during the first 12 hours, so that approximately a logarithmic order of death replaces the logarithmic order of growth, as was reported by Hobby, Meyer, and Chaffee (1942) and by Abraham and Duthie (1946).<sup>3</sup> For concentrations between 0.048 and 0.128  $\mu$ g per ml, the percentage of survivors at 12 hours was approximately the same, but at concentrations below 0.096  $\mu$ g per ml, the curves are V-shaped with an ascending branch from 12 hours on, evidencing a tendency toward restoration of the logarithmic order of growth, and corresponding to the "post-lytic waves of growth" reported by Bonét-Maury (1947) and Bonét-Maury and Pérault (1945) for cultures incubated with low concentrations of penicillin. At the optimal concentration (0.128  $\mu$ g per ml in this experiment), however, the approximately logarithmic order of death continued throughout the 48 hours of the experiment. With further increase in concentration the rate of killing was strikingly reduced, as shown by the higher level of the curve for 1.0  $\mu$ g per ml. The existence of an optimal concentration for maximal rate of killing is shown graphically in figure 2, which correlates the time required to kill 99.9 per cent of the test organisms with the concentration of penicillin.

Similar evidence of a maximal rate of killing with the minimal effective concentration of penicillin may be adduced also from the patterns that develop on assay plates processed by the 3-hour assay involving impregnation with silver and subsequent development (Goyan, Dufrenoy, Strait, and Pratt, 1947; Pratt, Goyan, Dufrenoy, and Strait, 1948). This technique is based on the principle that cells under the bacteriostatic influence of penicillin lose the ability, possessed by normal cells, to absorb silver nitrate. Thus, areas of the plate in which the

<sup>3</sup> For additional references see Pratt and Dufrenoy (1948).

cells have lost this ability appear clear following treatment with a solution of  $\text{AgNO}_3$ , exposure to light, and subsequent treatment with an appropriate developer, whereas areas of the plate containing cells that do absorb silver appear black

The outer extremity of a zone of inhibition on such plates corresponds to the site where penicillin in its outward diffusion from the cylinder has reached the critical concentration that causes maximal bacteriostatic effect with the shortest time of exposure (probably corresponding to the time between two successive cell divisions) This region is that of least density of silver, and appears as a



FIGS 1 and 2 SURVIVAL AT 37 C OF *STAPHYLOCOCCUS AUREUS* IN BROTH CONTAINING DIFFERENT CONCENTRATIONS OF PENICILLIN

Fig 1 Left Number of living organisms (expressed as percentage of initial inoculum) after different lengths of time Fig 2 Right Time required to kill 99.9 per cent of organisms (Note that concentrations of penicillin are expressed in  $\mu\text{g}$  per ml To convert to Oxford units per ml divide by 0.6)

clear ring on developed plates Immediately outside this ring, the concentration of penicillin attained is such as only to enhance metabolism and growth The deposition of silver is greatest in this region Immediately inside the clear periphery of the inhibition zone is a region in which, despite (or because of) the fact that there is a higher concentration of penicillin, deposition of silver occurs, indicating less rapid impairment of cellular activity than occurs in areas where cells are exposed to lower concentrations for a shorter period of time Thus, the test organisms that give the greatest response, as evidenced by physical development, are those that have been exposed for the shortest time to a minimal effective concentration It should be pointed out that the shortest time re-

ported by Eagle (1948) to accomplish 99.9 per cent killing in broth cultures of *S. aureus* was 5 hours, which corresponds approximately to the minimum time of total incubation required for latent inhibition zones to become demonstrable on assay plates seeded with the same organism.

#### MATERIALS AND METHODS

The present experiments were performed with cultures of *Staphylococcus aureus* NRRL 313 and of *Bacillus subtilis* NRRL B-558 in broths of the following compositions:

	<i>S. aureus</i>	<i>B. subtilis</i>
Difco peptone	6.0 g	5.0 g
N-Z case peptone <sup>4</sup>	4.0 g	—
Difco yeast extract	3.0 g	1.5 g
Difco beef extract	1.5 g	1.5 g
Glucose	1.0 g	1.0 g
Distilled water to	1,000 ml	1,000 ml

Cultures were inoculated with 1 ml of an 18-hour suspension of cells for 200 ml of broth, and were incubated at 37 C. Turbidity of the suspensions was estimated visually. The penicillins that were used were a crystalline sodium salt of benzyl penicillin that assayed 1,549 units per milligram by the cylinder plate method and a 9-amino-acridine penicillin prepared from crystalline potassium benzyl penicillin.<sup>5</sup>

#### EXPERIMENTS AND RESULTS

*Evidence for threshold and optimal concentrations of penicillin in broth.* The threshold concentration of penicillin required to check the growth of *S. aureus* in serial dilution tests was repeatedly found to be 0.01 unit per ml under the conditions of our experiments, whereas when the broth contained 1 mg of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  per liter the threshold occurred at 0.001 units per ml. We define the threshold in this paper as the minimal concentration of penicillin in which the broth remains clear for 48 hours. The threshold determined by observation of turbidity may be confirmed colorimetrically by the use of appropriate rH and pH indicators such as resazurin or phenolsulfon-phthalein, which give sharp end points in the range involved. These end points can also be defined by microscopical examination of drops removed from the several tubes and mixed with an equal volume of a 0.005 per cent solution of neutral red.

As cells of *S. aureus* come under the influence of toxic concentrations of penicillin, they swell and lose their tendency to form normal 3-dimensional colonies, instead they may line up in 2-dimensional "streptococcuslike" chains or they may simply form "diplococcuslike" cells that evidence bipolar staining due to

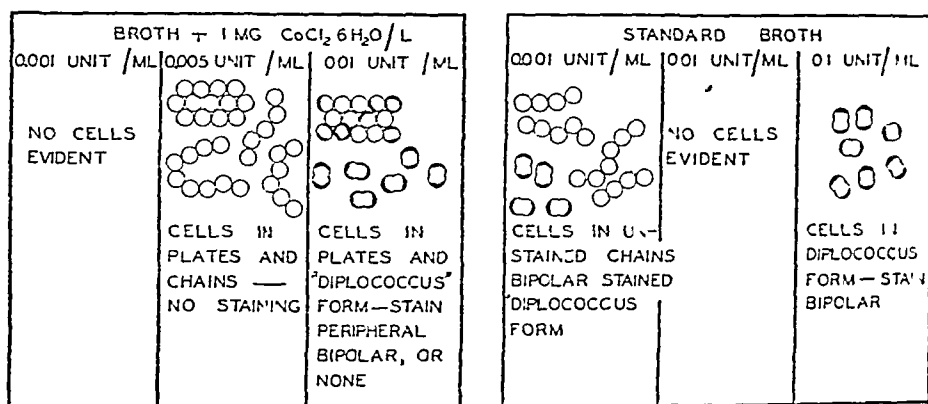
<sup>4</sup> Obtainable from Sheffield Farms Company, Inc., New York 19, New York.

<sup>5</sup> Prepared and kindly furnished by Dr. W. D. Kumler of this college. (Manuscript in preparation.)

displacement of the vacuolar material toward the periphery of the cell and its concentration at the poles (Pratt and Dufrenoy, 1947a) Concomitantly with these phenomena, the cells release their ribonucleic materials into the medium and lose their positive reaction to the gram stain

The morphological and cytochemical changes found in broth cultures are shown schematically in figures 3 and 4 The characteristic tendency of cells irreversibly damaged by penicillin to deteriorate progressively, losing their normal colonial habit of growth and becoming concatenated, at the same time losing the ability to absorb and concentrate neutral red in their vacuoles, was evidenced at lower concentrations in the cobalt series than in the control series, although the cells in both groups passed through the same sequence of events

The morphological evidence obtained tends to establish the validity of the concept of an optimal concentration of penicillin above which the antibacterial



FIGS 3 AND 4 DIAGRAMMATIC REPRESENTATION OF MORPHOLOGICAL AND STAINING CHARACTERISTICS OF CELLS OF *STAPHYLOCOCCUS AUREUS* EXPOSED FOR 18 HOURS AT 37 C TO DIFFERENT CONCENTRATIONS OF PENICILLIN IN BROTH

Fig 3 Left Standard broth plus 1 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}/\text{L}$  Fig 4 Right Standard broth

action is less rapid Figures 3 and 4, respectively, show that after 18 to 20 hours no cells were apparent in tubes with 0.001 unit per ml in the cobalt series or in tubes with 0.01 unit per ml in the controls, although cells in various stages of degeneration were evident in higher concentrations This suggests a slower rate of lysis, which may result in a lower over-all efficiency of penicillin in concentrations exceeding the minimal effective dose Several references in the literature point to the role of lysis products released into the medium in stimulating the metabolism of neighboring cells and thereby rendering them more susceptible to the deleterious action of penicillin (Bonét-Maury and Pérault, 1945, Abraham and Duthie, 1946, Bonét-Maury, 1947, Pratt and Dufrenoy, 1947b, 1948)

A direct correlation exists between the availability of oxygen and the ease with which the threshold concentration can be defined by turbidimetric, morphological, or chemical means This was shown by culturing the organisms in shallow layers where the diameter of the surface approximated the depth, and

in deeper layers where the depth was approximately 3 times the diameter of the surface. This observation confirms the earlier report of Mulé (1946), who reached the same conclusion by the use of different techniques.

Similar evidence of an optimal concentration for preventing growth has been found repeatedly when the 9-amino-acridine salt of penicillin has been tested against *Bacillus subtilis*. The results obtained by visual observations of turbidity in three representative tests are shown in table 1.

During the first 24 hours there was the conventional direct relation between concentration of drug in the broth and inhibition of growth of the organism. It is noteworthy, however, that longer periods of incubation revealed a distinct optimal concentration which completely checked proliferation, but below and above which growth occurred. The existence of the optimum was apparent

TABLE 1

*Growth of Bacillus subtilis in different concentrations of 9-amino-acridine benzyl penicillin*

CONCENTRATION OF ACRIDINE PENICILLIN IN PHOSPHATE BUF- FER (pH 6.9)	THEORETI- CAL PENICILLIN EQUIVA- LENT*	GROWTH† IN TUBES AFTER							
		18 Hours		24 Hours		42 Hours		62 Hours	
		Trial II	Trial III	Trial II	Trial III	Trial I	Trial II	Trial III	Trial I
	<i>u/ml</i>								
1 10 <sup>5</sup>	11 0	—	0	—	0	—	—	+	—
1 10 <sup>6</sup>	1 1	0	0	0	0	++++	++++	++	++
1 10 <sup>7</sup>	0 11	0	0	0	0	++	++	0	+++
1 5 × 10 <sup>7</sup>	0 022	0	0	0	0	++	0	0	+++
1 7.5 × 10 <sup>7</sup>	0 015	0+	0	++	0	0	+++	+	0
1 10 <sup>8</sup>	0 011	+	+	+++	+	—	++++	++	++
1 2 × 10 <sup>8</sup>	0 0055	—	+	—	++	—	—	+++	+++
1 5 × 10 <sup>8</sup>	0 0022	+++	—	++++	—	—	++++	—	—

\* Calculated from the quantities of potassium benzyl penicillin (purity 1,503 units per mg) and of 9 amino acridine that were reacted.

† 0 = no growth, tubes clear, ++++ = maximum turbidity, — means no observation made.

not only by visual observation of turbidity, but was confirmed colorimetrically by the use of triphenyl tetrazolium chloride as an indicator of pH and also morphologically and cytochemically by microscopic observation of drops removed from the several tubes and mixed with solutions of neutral red.

It is noteworthy that on the basis of penicillin content, the 9-amino-acridine salt of penicillin is 5 to 25 times more effective than the sodium salt in checking proliferation of *B. subtilis*. Whereas at least 0.06  $\mu$ g sodium penicillin (0.1 unit) per ml were required to inhibit proliferation to the eighteenth or twenty-fourth hours, and 0.3  $\mu$ g per ml were required to prevent a secondary wave of growth between the twenty-fourth to the forty-second hours, inhibition was obtained with as little as 0.015 to 0.02  $\mu$ g of any of the several lots of the 9-amino-acridine penicillin, embodying only 0.009 to 0.012  $\mu$ g of penicillin. The concentrations of the acridine penicillin that are involved are far below the inhibitory concentra-

tions of 9-amino-acridine alone. Thus, the effect noted here represents enhancement of penicillin activity and is not to be considered merely the result of two antibiotics acting simultaneously.

*Evidence for threshold and optimal concentrations of penicillin on assay plates*  
Since the preincubation technique, which permits cultures to reach the logarithmic phase of growth before coming under the influence of penicillin, makes it possible to expose the cells to the effect of diffusing penicillin at the phase of development when they are most reactive and the sharpest metabolic responses obtain, the sequence of events will be illustrated from seeded plates preincubated for 3 hours without penicillin and then reincubated for a second period of 3 hours dur-

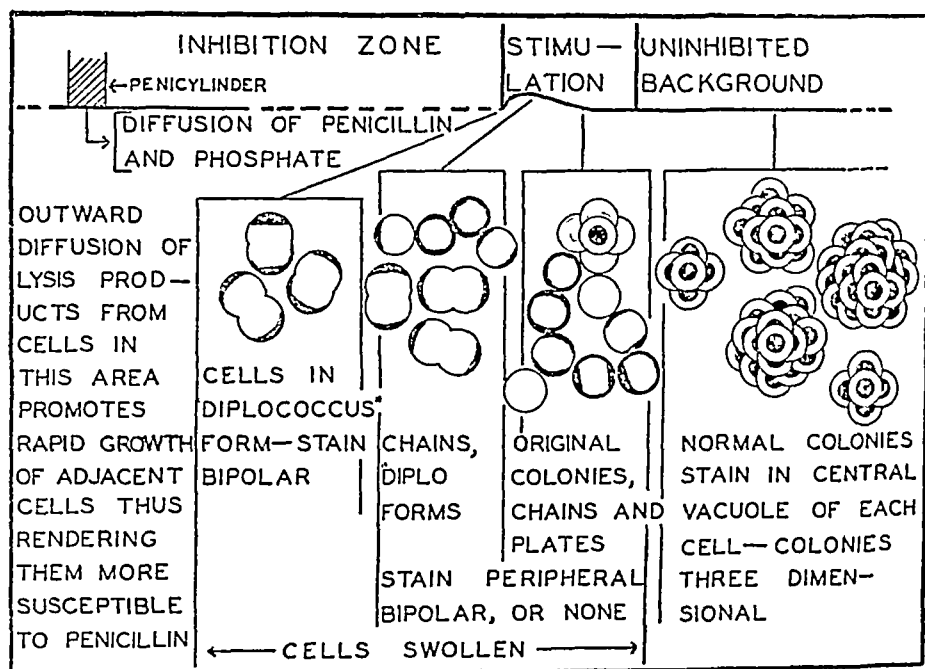


FIG 5 SCHEMATIZED DIAGRAMMATIC REPRESENTATION OF MORPHOLOGICAL AND STAINING CHARACTERISTICS OF CELLS OF *STAPHYLOCOCCUS AUREUS* IN DIFFERENT REGIONS OF A PENICILLIN ASSAY PLATE INCUBATED AT 37 C

ing which penicillin was diffusing from the cylinders. Figure 5 is a schematic representation of the morphological and vital staining characteristics of *S. aureus* in different parts of such plates. The figure (not drawn to scale) represents a portion of an inhibition zone and adjacent regions of the assay plate. The original micro colonies that form during the preincubation continue during the second incubation period to proliferate three-dimensionally in the background where they do not fall under the influence of enhancing or depressing concentrations of penicillin. The background is made up of randomly distributed colonies comprising living cells, each of which is able to absorb and concentrate neutral red or other vital dyes in its vacuolar solution (black in diagram). Where enhancing



concentrations of penicillin obtain, the original colonies no longer proliferate three-dimensionally, mostly within the agar, but instead the newly formed cells spread in one plane, forming "streptococcuslike" chains that may lie close together forming platelike structures on the surface of the agar. This alteration in spatial arrangement of the cells reflects a modification of the surface properties of the cells (to be discussed in terms of electrostatic charge in a subsequent paper). These changes probably are correlated with the release of cellular constituents from the swelling organisms, and can be made evident microscopically by the use of vital stains, since the staining, when it occurs, is peripheral, as shown under the outermost region of stimulation in the diagram.

Under slightly higher, though still subbacteriostatic concentrations, the cells become more loosely associated and finally resolve into "diplococcuslike" structures that evidence bipolar staining. It will be seen that the profile of the agar surface indicates a slightly raised level in the ring of stimulation (enhanced growth) wherein the material diffusing from the lysed organisms in the inhibition zone is actively metabolized. The tendency of the proliferating chains and plates of cells in this area to crowd the surface, as contrasted with the habit of the colonies in the normal background, may reflect an "oxygen hunger" induced in such cells. This is probably the result of two factors, i.e., direct stimulation by an appropriately low concentration of penicillin and an accelerated rate of metabolism induced by the availability in this region of "growth factors" released from the lysed cells in the inhibition zone.

The features described and diagrammed above can be observed *in situ*, under the oil immersion objective, on plates dehydrated with methylal<sup>6</sup> and then flooded with cedar oil.

The same sequence of changes in morphology and staining reaction of cells exposed to different concentrations of penicillin can be observed in serial dilution broth cultures. By analogy (compare figures 4 and 5) one might estimate the threshold concentration of penicillin delineating the ring of enhanced growth from the zone of inhibition on assay plates as being between 0.001 and 0.01 unit per ml. Incorporation in the agar of trace amounts of cobalt that are not bacteriostatic per se results in considerable enlargement of the zone of inhibition, indicating a lower threshold concentration of penicillin as compared to that obtaining on standard agar. By analogy with serial dilution tests (figure 3) the threshold on such plates may be estimated as below 0.001 unit per ml.

#### DISCUSSION

The effectiveness of cobalt in lowering the threshold concentration of penicillin for bacteriostasis *in vitro* and possibly *in vivo* (Pratt, Dufrenoy, and Strait, 1948) may be ascribed to formation of complexes involving —SH groups, although the possibility of other groups of cell proteins also being involved should not be overlooked. This is in accord with the conclusion of Barron (1944), that the effects of cobalt on living systems are manifestations of inhibition of —SH groups of

<sup>6</sup> Obtainable from Celanese Chemical Corporation, 180 Madison Avenue, New York City

enzymes, the functioning of which is so altered that the oxidation-reduction equilibria on which the survival of the cell depends are thrown irreparably out of balance

It is interesting to note in this connection that in our tests, which included Ir, Fe, Zn, Sr, Cd, Li, Cu, Ag, Au, and Bi, cobalt has been outstanding in lowering the bacteriostatic threshold of penicillin

As was pointed out by Young and Zelle (1946), to fulfill its function of coenzyme in the cell, glutathione must generally be in the reduced state (GSH), since the presence of —SH groups is necessary for the activity of such enzymes as glyoxalase, succinic dehydrogenase, phosphorylase, etc. These authors in studying the respiratory pathogenicity of *Bacillus anthracis* spores pointed out that the presence of heavy metals markedly accelerates the oxidation of reduced glutathione to the disulfide state

It should be pointed out that the phenomenon of occurrence of an optimal range or ranges of concentration that check metabolic activity of test organisms, but above and below which normal or even accelerated activity may occur, is not peculiar to penicillin. A similar phenomenon has been reported by Welch, Price, and Randall (1946) for streptothricin tested against *S. aureus* and for streptomycin tested against two strains of *Eberthella typhosa*. They noted that "relatively high concentrations of streptothricin did not interfere with the ability of the organism to reduce nitrate to nitrite, while somewhat lower concentrations caused complete inhibition of nitrate reduction," and that "essentially the same results were obtained when streptomycin was substituted for streptothricin."

Moreover, such a phenomenon is not restricted to the action of antibiotics. That a given concentration of a metal may adversely affect measurable manifestations of metabolism more effectively than either lower or higher concentrations was considered a "general law" by Richet as early as 1906. It is pertinent to note that his results were obtained from a study of the effect of different concentrations of metals, including cobalt, on lactic acid fermentation, which is now known to involve glyoxalase, the activity of which depends on —SH groups.

#### ADDENDUM

Just as the proof of this paper was received, an additional pertinent report showing the existence of optimal concentrations of penicillin for antibacterial action came to hand.

Eriksen (1946), working with *S. aureus*, observed that "the highest degrees of lysis occur in the weaker concentrations (1/16–1/64 unit per cc). In the strong concentrations (1–1/2 unit) lysis is very insignificant." He also noted that cells exposed to weak concentrations of penicillin undergo marked changes in morphology but that "in strong concentrations of penicillin (1–1/2 unit per cc) there is no corresponding alteration in the morphology."

#### SUMMARY

Employing standard serial dilution techniques, it was found that the presence of trace amounts of cobalt in the broth markedly lowered the concentration of penicillin required to inhibit proliferation of *Staphylococcus aureus*. Concomi-

tant tests with suitable rH and pH indicators confirmed the "end point" concentrations that were determined on the basis of turbidity. Microscopical examination of cells from the several cultures showed that in the controls and in the cobalt series exposed to penicillin the same morphological and cytochemical changes occurred, viz., a tendency to change from the normal colonial habit of growth to the formation of "streptococcuslike" chains of swollen cells which may become arranged in plates and finally separate to form "diplococcuslike" structures that evidence bipolar staining with vital dyes instead of the normal tendency to accumulate such dyes in the central vacuolar material.

Similar phenomena were demonstrated on assay plates exposed to penicillin.

Evidence based on observations of turbidity, of response of the cultures to rH and pH indicators, and morphological changes in the cells, indicates the existence of an optimum concentration of penicillin above and below which inhibition of cellular activity is less pronounced.

Similar evidence of an optimum concentration was obtained in tests of the 9-amino-acridine salt of penicillin tested against *Bacillus subtilis*.

An approximation is made of the concentration of penicillin at the site of the periphery of inhibition zones on assay plates seeded with *S. aureus*.

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# GRISEIN, AN ANTIBIOTIC PRODUCED BY CERTAIN STRAINS OF *STREPTOMYCES GRISEUS*<sup>1 2</sup>

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It has now been definitely established that the ability to produce streptomycin is limited to a certain few strains of *Streptomyces griseus*. Numerous other strains of this organism, either isolated from natural substrates or taken from culture collections, were found to be unable to produce streptomycin or any other antibiotic. A certain few strains, however, were found capable of forming other antibiotics (Waksman, Schatz, and Reynolds, 1946). One such strain produced in the culture medium an antibacterial substance that was designated "grisein"; this antibiotic was characterized by certain chemical and antibacterial properties that were quite distinct from other well-known antibiotics (Reynolds, Schatz, and Waksman, 1947).

Although grisein is in some respects similar to streptothricin and to streptomycin, such as in its solubility in water and its selective activity against various gram-positive and gram-negative bacteria, it is strikingly different from these two antibiotics in other respects. Grisein is adsorbed from the medium on activated charcoal, it is not eluted from the adsorbent by alcoholic acid solutions, but by neutral 95 per cent ethanol. Grisein has a much narrower antibacterial spectrum than that of streptothricin and streptomycin. It is not active, for example, upon *Mycobacterium tuberculosis* or upon other bacteria sensitive to the other two antibiotics. Bacteria develop resistance to grisein much more rapidly than to either of the other antibiotics.

## EXPERIMENTAL PROCEDURES AND RESULTS

*Description of organism* The grisein-producing strain of *Streptomyces griseus* (Klansky) Waksman and Curtis shows the major characteristic properties of the species, and can scarcely be distinguished from the streptomycin-producing strains of this organism or from the inactive strains.

The following are the characteristic properties of *S. griseus*: (1) cream-colored growth on organic and synthetic media, (2) white to cream-colored aerial mycelium with light-greenish tinge, (3) formation of aerial hyphae in clusters or tufts, the hyphae being straight and without spirals (figure 1), (4) lack of pigment formation on potato, gelatin, and various peptone and protein-containing media, and (5) strong proteolytic activities, as characterized by rapid liquefaction of gelatin.

<sup>1</sup> Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

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If there are any distinguishing characteristics that would differentiate between the grisein-producing and the other strains of *S. griseus*, they are to be looked for

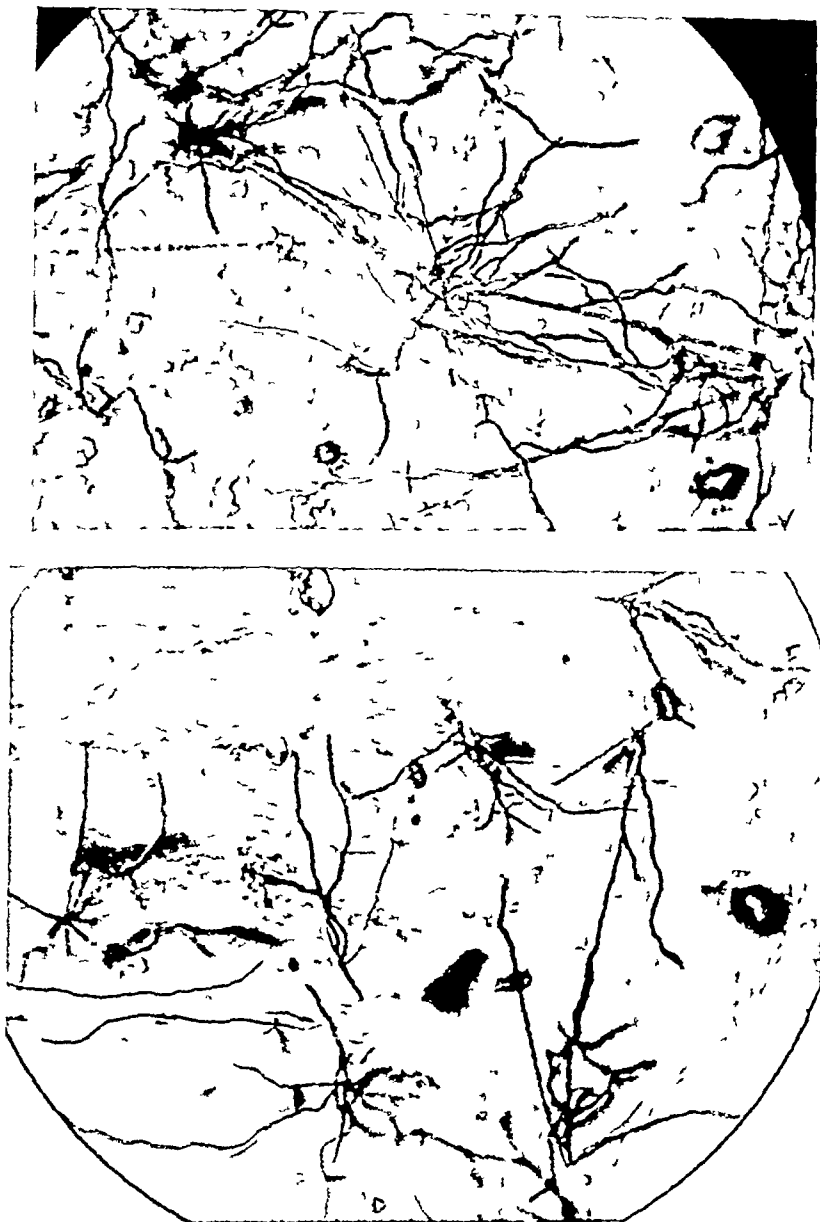


FIG. 1. FORMATION OF AERIAL MYCELIUM BY A GRISEIN-PRODUCING STRAIN OF *S. griseus*.

Note characteristic formation of aerial hyphae arising either from a central point or knot or close to one another. Upper: Abundant hyphae. Lower: Few hyphae.

in some of the biochemical activities. So far, two important differentiating properties have been established: first, the formation of the particular antibiotic,

that is, the ability to produce grisein, which is not formed by other cultures, and, second, the nonsensitivity of the grisein-producing cultures to phage, the streptomycin-producing strains being sensitive to a specific actinophage (Reilly, Harris, and Waksman, 1947). There is another property that is more one of degree than of kind, namely, a lesser tendency of the aerial mycelium to show the typical grass-green coloration, the pigmentation remaining more cream-colored. This property is largely influenced, however, by the composition of the medium.

*Production and testing of grisein.* The methods of growing the organism for the production of grisein are similar to those commonly used for streptomycin. A simple nutrient broth, consisting of 0.5 per cent peptone, 0.3 per cent meat extract, and 0.3 per cent NaCl, was at first used. Glucose was found to exert an initial delaying effect upon the production of grisein. Later, more effective media were developed. The medium is inoculated with spores of the grisein-producing strains of *S. griseus* (no. 3478), and the cultures are incubated, in a stationary condition, for 6 to 12 days or in a shaken or submerged condition for 4 to 6 days.

The antibacterial potency of the culture filtrate and of the isolated crude grisein preparations can be determined either by the agar streak dilution method, or by the agar diffusion or cup method. The rapid development of bacterial cells resistant to grisein precludes the use of serial dilution procedures in the liquid media.

A streptomycin-resistant strain of *Escherichia coli* gives the best results as a test organism by the cup method of assay; grisein produces a very clear zone with this organism. Such a strain can further be used for differentiating between grisein and streptomycin, and thus give measurements for the concentration of grisein in the medium even in the case of cultures that may also produce some streptomycin. In the cup assay method, grisein gives identical zones whether a streptomycin-sensitive or a streptomycin-resistant culture of *Escherichia coli* is used. In contrast to streptomycin, the activity of grisein is unaffected by the presence of glucose in the test medium, either in the agar streak method or in the cup method.

In testing for the concentration of grisein, a 24-hour-old broth culture of *E. coli* is used to inoculate the nutrient agar. The inoculation and pouring of the plates, the preparation of the cups, the incubation of the plates, and the reading of the zones are conducted in accordance with the procedures developed for assaying penicillin and streptomycin. As in the case of the latter, the values obtained by the cup assay method are a function of the pH at which the assay agar is buffered.

*Formation and isolation of grisein.* When first tested by the cross-streak method on agar plates, *S. griseus* 3478 was found to exert the greatest activity against certain gram-negative bacteria, notably *E. coli*, and against certain gram-positive organisms, notably *Bacillus subtilis* and *Staphylococcus aureus*, as shown in table 1. The culture was much less active against most other bacteria, such as *Serratia marcescens*, *Pseudomonas aeruginosa*, *Sarcina lutea*, and *Bacillus mycoides*, with no activity at all against various other bacteria, including *Klebsiella pneumoniae* and *Aerobacter aerogenes*.

When *S. griseus* 3478 was grown in nutrient broth, the culture filtrate was found to contain an antibiotic substance that had a spectrum even narrower than that shown by the growing culture in the cross-streak method. These results, presented in table 2, fully confirm those reported previously (Reynolds, Schatz, and Waksman, 1947). The active substance, namely the grisein, had no effect at all against a number of bacteria found both among the gram-positive (*Bacillus mycoides*, *Sarcina lutea*, and *Mycobacterium phlei*) and gram-negative (*Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Proteus vulgaris*) groups. It was not active against fungi. The discrepancy between the results obtained by the cross-streak method and the activity of the substance produced in the liquid culture medium

TABLE 1

The antagonistic activity of *Streptomyces griseus* 3478 against various bacteria  
(Cross streak plate method\*)

ORGANISM	ZONE OF INHIBITION mm
<i>E. coli</i>	16
<i>P. aeruginosa</i>	5
<i>P. fluorescens</i>	7
<i>S. marcescens</i>	7
<i>K. pneumoniae</i>	0
<i>A. aerogenes</i>	0
<i>B. subtilis</i>	12
<i>B. megatherium</i>	6
<i>B. mycoides</i>	5
<i>S. aureus</i>	13
<i>S. lutea</i>	2
<i>M. phlei</i>	6
<i>M. tuberculosis</i> 607	3

\* Plates containing 14 ml nutrient agar were streaked with no. 3478, and incubated 48 hours at 28 C. The plates were then cross streaked with various bacteria and incubated for 20 hours at 28 C, the *M. phlei* and *M. tuberculosis* plates were incubated at 37 C for 2 days. All figures represent averages of 10 bacterial streaks.

suggests the probability that under certain conditions of cultivation another antibiotic distinct from grisein may be produced by the organism.

The addition of glucose to the medium was found to delay grisein production. This was due to a reduction in the pH of the medium because of the production of acid from the glucose. When the glucose medium was buffered with phosphate at pH 7.0, grisein was produced in smaller amounts, however, than in the unbuffered medium or in simple nutrient broth.

The addition of iron salts to organic or to synthetic media, such as a modification of Thornberry's medium (1946), had a strikingly favorable effect upon the antibiotic activity of the culture filtrate and upon the yield of grisein. This is brought out in figure 2. Equivalent amounts of iron, in the form of ferrous sulfate, ferric ammonium citrate, and peptonized iron, brought about approximately



the same stimulation of grisein production. The antibiotic substance isolated from the media that were supplemented with ferrous salt gave the same antibacterial spectrum as the grisein preparations obtained from media not enriched with iron, thus indicating that the increase in activity was due to an actual increase in the formation of grisein.

In synthetic media, ammonium citrate proved to be superior as a source of nitrogen when compared to other compounds, with glycine coming next. When iron was present, the activity of the synthetic broth was increased from 0 to 2,200 units per ml or even higher.

In order to isolate crude grisein preparations the metabolite solution was filtered through glass wool and filter cel, and treated with 8 g of norit A per liter. The grisein was adsorbed on the norit. The adsorbent was removed by filtration,

TABLE 2

*Antibiotic spectrum of a culture filtrate of S. griseus 3478*

(Incubated, under submerged conditions of growth, for 4 days, and tested by agar plate streak method)

ORGANISM	DILUTION UNITS/ML
<i>E. coli</i> W	250
<i>E. coli</i> 2	250
<i>A. aerogenes</i>	0
<i>S. marcescens</i>	250
<i>P. aeruginosa</i>	0
<i>P. fluorescens</i>	0
<i>P. vulgaris</i>	0
<i>B. subtilis</i>	200
<i>B. mycoides</i>	0
<i>B. megatherium</i>	100
<i>S. aureus</i>	>300
<i>S. lutea</i>	0
<i>M. phlei</i>	0
<i>P. notatum</i>	0
<i>T. mentagrophytes</i>	0

and treated with 95 per cent ethanol, 10 ml per gram. The resulting slurry was stirred for 2 hours at 45 C, and the brownish ethanolic eluate separated by filtration. The filtrate was reduced to approximately one-fiftieth the original volume by vacuum distillation at 55 C. Methanol was added to the remaining syrup until a precipitate just began to form, and the suspension was added dropwise, with constant stirring, to acetone, the active substance came out in the form of a flocculent precipitate. This precipitate was desiccated *in vacuo* and stored at 6 C. Nonsterilized grisein solutions stored at 6 C favored the growth of contaminating fungi and bacteria much more frequently than is the case with streptomycin, because of the narrower antibiotic spectrum of the grisein.

The results of a typical experiment, using the foregoing procedure, may be cited. 11.5 liters of metabolite solution obtained by growing the organism in

shaken culture for 4 days at 28 C gave a yield of 2.67 g of crude grisein. The culture tested 700 units per ml, the resulting product tested 430 units per mg. The yield of the antibiotic was, therefore, 14.5 per cent of the theoretical.

*Chemical properties of grisein.* Crude grisein preparations are completely soluble in water, slightly soluble in 95 per cent ethanol, and insoluble in absolute ethanol, ether, and acetone. The activity remains unchanged even when the solution is heated to 100 C for 10 minutes. When grisein is treated for 20 hours with 0.2 N formic acid in 50 per cent aqueous methanol (pH 2.2), followed by neutralization of the solution with an alkali, all the grisein is recovered.

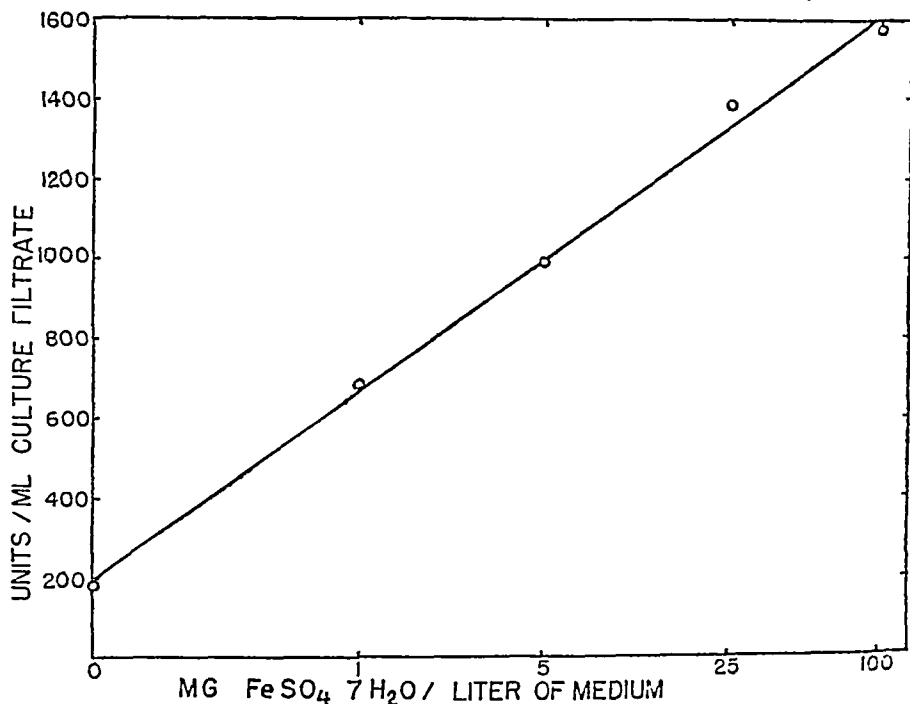


FIG. 2. THE EFFECT OF IRON AS FERROUS SULFATE ON THE PRODUCTION OF GRISEIN.

Recorded values are peaks of activity obtained with meat extract peptone NaCl medium in submerged culture.

When the culture filtrate is treated with filter cel, or alumina at pH 4.5, or when it is passed through a Berkefeld filter, none of the grisein is adsorbed.

Grisein is sensitive to increasing acidity of the substrate, in a manner similar to that of streptomycin, as shown in figure 3. Grisein differs from streptomycin in that it remains unaffected by sulfhydryl compounds or by carbonyl group reagents (table 3). The various preparations were made up in concentrations just below the levels required to inhibit the growth of *E. coli* and were added to appropriate aliquots of stock solutions of the two antibiotics. The antibacterial activity of the mixtures was measured by the agar cup diffusion method using *E. coli* as the test organism and a standard grisein preparation. Whereas strepto-

mycin is readily inactivated by cysteine, hydroxylamine, and certain other reagents, these have little if any effect upon the potency of grisein

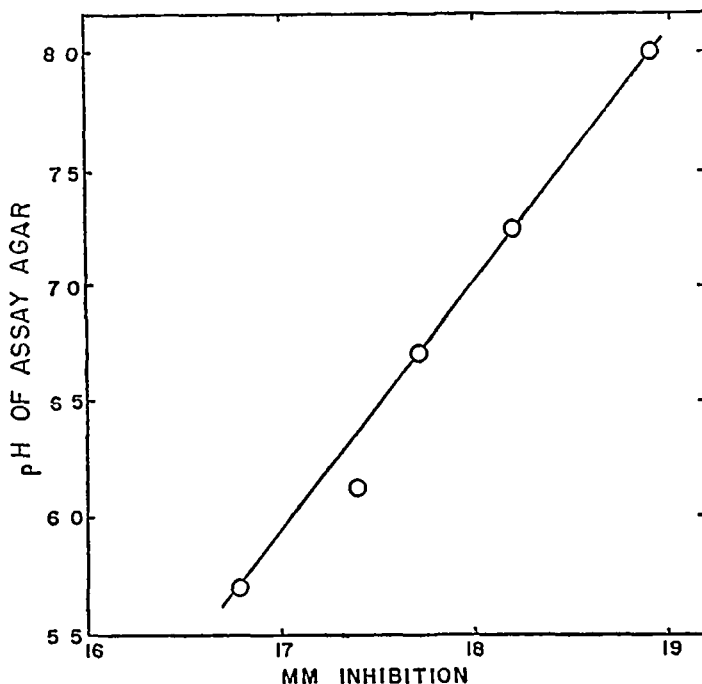


FIG 3 EFFECT OF pH OF NUTRIENT ASSAY AGAR ON ZONE SIZE OBTAINED IN CUP DIFFUSION ASSAY OF GRISEIN WITH *E. COLI*

TABLE 3

*The effect of sulfhydryl compounds and carbonyl group reagents on the antibacterial activity of grisein and streptomycin*

REAGENTS 0.0013 M SOLUTION*	INHIBITION OF <i>E. COLI</i> ZONES IN MM†		
	Streptomycin		Grisein
	16 µg/ml	25 µg/ml‡	200 units/ml
Control	15.8	27.0	16.1
Cysteine	10.4	8.0	14.7
Sodium thioglycolate	15.6	25.0	16.1
Hydrazine	10.7	10.0	15.8
Hydroxylamine	10.0	8.0	15.8
Semicarbazide	10.0	—	16.4

\* Cysteine is 0.013 M

† Assay cup has a diameter of 8 mm

‡ These results were obtained in an earlier experiment

*Antibacterial properties of grisein* Just as in the case of the results obtained by the cross-streak method and in the metabolite solution, isolated and concen-

trated grisein preparations possess relatively narrow antibacterial spectra. This is brought out in table 4. Numerous gram-negative and gram-positive bacteria, including the acid-fast bacteria, are not sensitive to it. Certain bacteria that were originally sensitive to the crude culture filtrate became nonsensitive to the isolated grisein, this is true, for example, of *Serratia marcescens*. When a low potency preparation was tested by the turbidimetric method against the non-pathogenic *Mycobacterium tuberculosis* 607, using the modified Dubos "tween 80" medium, no inhibition was obtained. Grisein is highly active, however, against various species of *Salmonella* and *Shigella*, and against *Micrococcus lysodeikticus*.

In the early studies of streptomycin it was found that a streptothricin standard could be used for standardization purposes. Unfortunately, a similar standard

TABLE 4  
The antibacterial spectrum of a potent preparation of grisein  
(Units of activity per mg of preparation)

<i>E. coli</i>	1,800
<i>S. marcescens</i>	<100
<i>P. aeruginosa</i>	<100
<i>P. fluorescens</i>	<30
<i>A. aerogenes</i>	<30
<i>K. pneumoniae</i>	<30
<i>P. vulgaris</i>	<30
<i>S. pullorum</i>	30,000
<i>S. dysenteriae</i>	18,000
<i>S. paradysenteriae</i>	18,000
<i>S. alkalescens</i>	1,800
<i>M. tuberculosis</i> 607	<30
<i>M. phlei</i>	<30
<i>B. subtilis</i>	300
<i>B. mycoides</i>	<30
<i>B. megatherium</i>	1,000
<i>B. cereus</i>	1,000
<i>M. lysodeikticus</i>	30,000
<i>S. aureus</i>	3,000
<i>S. lutea</i>	<30

cannot be used with great accuracy for measuring the activity of crude grisein preparations. This is because the slopes of the curves for the two antibiotics differ slightly. In addition to the reason given previously for using *E. coli* for standardizing grisein preparations, there was also the fact that by the cup method this antibiotic allows the formation of an excellent zone of inhibition of *E. coli*, as compared to a very undefined, narrow zone produced by streptomycin. *B. subtilis*, which is commonly used in testing streptomycin preparations, gives a sharp zone with grisein, but the rapid development of resistant strains against this antibiotic makes accurate readings difficult. When *S. aureus* is used as the test organism against a streptomycin standard, grisein preparations give a higher unitage than that obtained with either of the foregoing two test bacteria. This

is brought out in table 5. The zones of inhibition were generally lower at 37 C than at 28 C, they were sharper, however, and could be read more easily on the plates incubated at the higher temperature. Streptomycin gave narrower zones against *E. coli* than did grisein. That was the chief reason for adopting *E. coli* as the test organism and for measuring grisein activity in terms of *E. coli* dilution units against a given standard.

Streptomycin-resistant cultures of *E. coli* and *S. aureus* were frequently more sensitive to grisein than were the parent cultures. The Bodenheimer organism,

TABLE 5

*The influence of the temperature of incubation upon growth inhibition of bacteria by streptomycin and grisein*

TEMPERATURE (C)	ANTIBIOTIC	CONCENTRATION	ZONE OF INHIBITION MM		
			<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>
28	Streptomycin	50 µg/ml	14.8	24.8	18.8
37	Streptomycin	50 µg/ml	13.4	19.5	17.0
28	Grisein	4 mg/ml	19.0	20.5	28.5
37	Grisein	4 mg/ml	18.1	14.3	21.5

TABLE 6

*The effect of iron on the activity of grisein*

ASSAY	IRON * MG/L AGAR	<i>E. COLI</i> , U/ML
Agar streak	0	1,000
Agar streak	50	900
Agar streak	500	0
		ZONE OF <i>E. COLI</i> INHIBITION MM
Cup method	0	16.6
Cup method	70	16.6
Cup method	104	8.0

\* FeSO<sub>4</sub> 7H<sub>2</sub>O

however, which is resistant to streptomycin and sensitive to streptothricin, was also found to be resistant to grisein.

*Effect of iron on grisein activity.* In spite of the fact that the presence of iron in the medium greatly stimulates the production of grisein, it exerts an inhibitory effect on the activity of this antibiotic. This effect can be demonstrated when both the iron salt and the grisein preparations are incorporated into the nutrient agar assay medium and the plate is streaked with the test bacteria, or when the iron salt is incorporated in the medium and the grisein solution is placed in cups. The results are presented in table 6. As little as 0.0004 M ferric iron completely inhibited the antibacterial effect of grisein upon *E. coli*.

To determine whether the iron ion is responsible for the inactivation of the grisein, three different concentrations of the sulfates of seven cations were incorporated into various lots of nutrient agar. Certain deviations in the inhibition activity of the grisein were produced by the various cations as compared to the control medium, however, only those media to which ferrous sulfate was added showed complete lack of grisein activity.

When iron compounds were added to a solution of grisein and tested by the cup method, an inactivating effect was also obtained, much higher concentrations of iron being required, however, to bring this about. The addition of 4 g of ferrous sulfate per liter partly inactivated grisein, whereas 25 g per L brought about its complete inactivation. Three grams of ferric ammonium citrate per liter gave partial inactivation, but no complete inactivation was obtained even by 100 g per L of this salt.

This inactivating effect on grisein by iron may be due to the formation of an insoluble iron-grisein complex. Similar effects were observed in the study of the inactivation of aspergillic acid by iron (Goth, 1945). No precipitate was obtained when aqueous solutions of grisein and of ferrous sulfate were mixed, when the iron was removed from such solutions with hydrogen sulfide and alkaline acetate, and the  $H_2S$  evaporated over KOH, the activity of the grisein was restored. Thus, if an insoluble complex is formed, the process can be reversed under the conditions noted.

*Development of resistance to grisein.* When culture media containing grisein are assayed by the agar streak dilution procedure, colonies of the test organisms, notably of *E. coli* and *S. aureus*, resistant to grisein often appear in the streaked areas. The resistant nature of the strains thus obtained was confirmed by picking the colonies and growing them on grisein-containing agar. The greater frequency with which resistant colonies appear in streak assay procedures with grisein indicated that the development of resistant strains was much more rapid against this antibiotic than against streptomycin.

This tendency of grisein to favor the formation of resistant strains was further brought out in studies of its action on *E. coli* in nutrient broth cultures. The growth curves obtained in the presence of several concentrations of grisein show no bactericidal effect. A bacteriostatic action was observed only during the first 6 hours of incubation, after which growth proceeded as rapidly as in grisein-free cultures. It is this property of grisein that precluded its assay by the use of broth dilution procedures. A comparison of the growth of *E. coli* in nutrient broth containing grisein or streptomycin showed (table 7) that, after 48 hours' incubation, more than 10 times the number of resistant cells were present in the grisein-containing than in the streptomycin-containing medium.

The difference in the action of the two antibiotics was most strikingly demonstrated when 20-hour *E. coli* cultures in nutrient broth were plated out in nutrient agar media containing increasing concentrations of the individual antibiotics and with increasing levels of one antibiotic plus a fixed concentration of the other agent. The effect of different concentrations of streptomycin results in a sharp

drop in the numbers of viable bacterial cells between 1 and 4 units per ml, when the amount of streptomycin is increased to 15 units per ml no resistant cells are left. In the case of grisein, however, the cell count drops to half the control

TABLE 7  
*The growth of E. coli in media containing grisein and streptomycin*

ANTIBIOTIC	UNITS/ML	CELLS/ML AFTER 48 HR INCUBATION AT 23 C $\times 10^8$
Control	0	800
Streptomycin	1	250
	3	3
Grisein	10	40
	50	60
	250	60

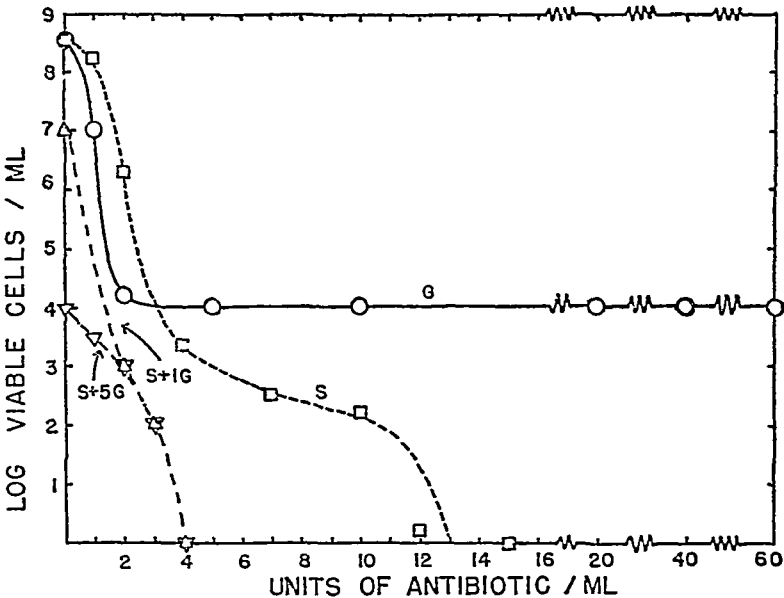


FIG 4 INFLUENCE OF CONCENTRATION OF ANTIBIOTICS IN AGAR MEDIA AND THE DEVELOPMENT OF RESISTANCE BY *E. COLI*

- Streptomycin
- Grisein
- △ Streptomycin + 1 unit of grisein per ml
- ▽ Streptomycin + 5 units of grisein per ml

population with 2 units per ml, and thereafter levels off to a constant value. This is brought out in figure 4.

If fixed amounts of grisein (1, 5, and 10 units per ml) are added to the plating

agar containing increasing amounts of streptomycin, the resulting curves suggest an additive effect in low concentrations, and a synergistic effect at higher levels. Thus, 1 unit per ml grisein plus 2 units per ml streptomycin (totaling 3 units per ml) gave  $1 \times 10^3$  cells per ml when 1 ml of a 20-hour-old culture of *E. coli* was plated out, whereas 3 units per ml of streptomycin gave  $1 \times 10^4$  cells per ml. At higher concentrations, namely, when 1 unit per ml grisein was added to 5 units per ml streptomycin (totaling 6 units per ml), no resistant cells developed, whereas the corresponding agar containing 6 units per ml of streptomycin gave a cell count of  $1 \times 10^3$  per ml. Conversely, if fixed amounts of streptomycin (1 and 2 units per ml) are added to agar containing increasing amounts of grisein, the

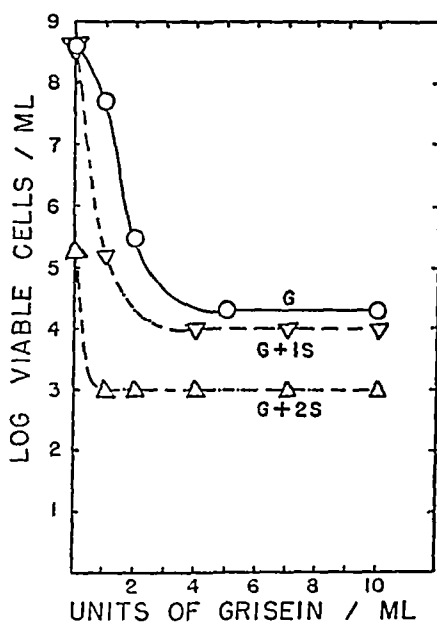


FIG. 5. EFFECT OF VARYING CONCENTRATIONS OF ANTIBIOTICS IN NUTRIENT AGAR UPON THE DEVELOPMENT OF RESISTANCE BY *E. COLI*.

- Grisein alone
- ▽ Grisein + 1 unit of streptomycin per ml
- △ Grisein + 2 units of streptomycin per ml

effect is merely additive, regardless of the concentration of grisein used (figure 5). Thus 1 unit per ml streptomycin plus 2 units per ml grisein (totaling 3 units per ml) gave  $3 \times 10^4$  residual cells per ml, 3 units per ml grisein giving  $5 \times 10^4$  cells per ml. At the higher levels, 1 unit per ml streptomycin plus 5 units per ml of grisein (totaling 6 units per ml) gave a count of  $1 \times 10^4$  cells per ml, 6 units per ml grisein giving  $3 \times 10^4$  resistant cells per ml. Hence, though it was possible to prevent the detectable development of resistant cells by adding 1 unit per ml of grisein to 4 units per ml of streptomycin,<sup>3</sup> such growth could not be prevented by

<sup>3</sup> Sixteen units per ml of streptomycin itself would be required to prevent detectable development of resistant cells under these conditions.



the addition of small concentrations of streptomycin to high concentrations of grisein

*In vivo activity of grisein* The rapid development of strains of bacteria resistant to grisein leads one to hold in abeyance any evaluation of the potentialities of this antibiotic as a chemotherapeutic agent. Nevertheless, preliminary studies made by Mr Otto Graessle, at the Merck Institute for Therapeutic Research, indicated that grisein is well tolerated by experimental animals even in concentrations of 500,000 units per kg of body weight. Mice were protected against *Salmonella schottmülleri* and *S aureus* infections with doses of grisein ranging from 800 to 1,600 units per mouse. Grisein may, therefore, prove to be of value in supplementing streptomycin therapy, for the purpose of suppressing the development of resistant strains of certain gram-negative bacteria against which it is effective.

#### SUMMARY

Grisein, an antibiotic distinct from streptomycin, is produced by certain strains of *Streptomyces griseus*, when grown in organic or in synthetic media, in stationary or in submerged culture.

Grisein concentration in the metabolite solution is best measured by using a streptomycin-resistant strain of *Escherichia coli*, in order to avoid possible contamination with any streptomycin that the organism may produce.

The production of grisein is greatly favored by the presence in the medium of iron compounds. The addition of iron salts to the test medium, however, reduces considerably the antibacterial potency of grisein preparations.

Although grisein is similar, in its solubility and in its general antibacterial properties, to streptomycin and streptothricin, it differs from these antibiotics by a lack of basic properties and by a much narrower antibiotic spectrum.

Grisein is resistant to heat (100 C for 10 minutes) and to acid treatment at room temperature. The activity of grisein depends upon the hydrogen ion concentrations in the medium, an effect similar to that of pH upon streptomycin.

The activity of grisein is not affected by sulfhydryl or carbonyl group reagents.

Cultures of *E coli* and of *Staphylococcus aureus* made resistant to streptomycin and to streptothricin are sensitive to grisein. Other streptomycin-resistant bacteria, like the Bodenheimer organism, are also resistant to grisein.

The addition of small amounts of grisein to streptomycin solutions produces a synergistic effect on bacteria sensitive to both antibiotics, the development of resistant bacteria being greatly depressed.

Grisein possesses only a limited toxicity to experimental animals, and is active *in vivo* against bacteria sensitive to it *in vitro*.

The authors wish to express their sincere appreciation to Dr Dorothy Smith and to Dr H Christine Reilly, of this laboratory, for assisting with the testing of various grisein preparations, and to Mr O Graessle, of the Merck Institute for Therapeutic Research, for conducting the *in vivo* experiments and for permitting us to refer to his data in this paper.

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# PRELIMINARY OBSERVATIONS ON GERMINATION OF THE SPORES OF *BACILLUS MYCOIDES* IN A NITROGEN-FREE MEDIUM AND CERTAIN PROPERTIES OF THE TRANSPARENT CELLS

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The ability of the endospores of *Bacillus mycoides* to germinate in a medium free of nitrogen, but containing a source of energy, was recently shown by Knaysı (1945). This was confirmed by Knaysı and Baker (1947), who also observed germination in solutions of glucose and sodium acetate, showing that the endospore contains a source of both nitrogen and phosphorus. This and other considerations (Knaysı, 1946) indicated that the spore contains a relatively large quantity of ribonucleic acid. Search of the literature to ascertain whether spore germination under similar conditions had previously been observed led only to a parenthetical statement by Eijkman (1918) indicating that he observed endospore germination in solutions of glucose, lactose, and mannitol, but not in solutions of glycerol. In the case of *Bacillus mycoides*, Knaysı (1945) showed that lactose is inactive and pointed out that germination is induced only by substances which can be utilized as a source of energy. He also pointed out the importance of buffering the solution. It should be emphasized, however, that the proportion of spores which germinate under these conditions is variable and usually small, and that many of the vegetative cells observed develop as a result of growth. In view of the value of the vegetative cells which develop in such media for cytological investigations, and of their potential value in other fields of theoretical and practical bacteriology, it was found desirable to study the conditions which control spore germination in nitrogen-free media.

## SCOPE, STRAIN, AND TECHNIQUE

In the present work we did not study the effect of the medium in which the spores are formed. In all experiments the spores of *Bacillus mycoides*, C<sub>2</sub>, were harvested from slant cultures on the medium: meat infusion,  $\frac{1}{4}$  strength, 100 ml, tryptone, 0.25 g, glucose, 0.25 g, and agar, 1.5 g. The growth was very carefully scraped off two 7-ml slants and washed three times, each time in 10 ml of sterile, distilled water, and finally suspended in 10 ml of sterile, distilled water. When the cultures were 6 or more days old at 27 to 28 C, the suspension was practically free of vegetative cells (<1 per cent). Since germination in the media used was slow, we recorded the proportions of vegetative cells and spores in cultures 16 to 24 hours old at 33 C. The media were inoculated heavily ( $4 \times 10^8$  to  $20 \times 10^8$  spores per ml).

The medium used most in this work was a solution of 0.2 g of glucose and 0.2 g of sodium acetate in 100 ml of distilled water. This medium was often supple-

mented with small amounts of an equimolar mixture of monopotassium and dipotassium phosphates varying from 1 to 10 parts in 2,000 parts of the medium. In a few experiments the medium was supplemented, in addition, with other mineral salts (0.1 ml of Speakman's mixture B which had been diluted 100-fold to 5 ml of the medium), or with biotin (3  $\mu$ g per ml of medium).

#### RESULTS

The results of numerous experiments led to the following conclusion. The number of vegetative cells that develop in the glucose, acetate solution depends within wide limits, on the age of the culture from which the spores were harvested and, particularly, on the age of the spore suspension. In one typical experiment, for instance, a spore suspension freshly prepared from a slant culture 24 days old at 25 to 27 C gave 20 per cent of vegetative cells after 16 hours of incubation, 23 days later the same suspension gave 2.4 per cent. In both cases the inoculum was  $15 \times 10^7$  spores per ml. The addition of ammonium sulfate to the medium did not increase the number of vegetative cells when the medium was inoculated from the old suspension.

We also made the interesting observation that the number of vegetative cells that develop in the glucose, acetate medium may be increased when the stock solutions from which the medium is prepared (2 g of glucose and 2 g of sodium acetate, each in 100 ml of distilled water) are freshly autoclaved. The effect of autoclaving persists for a number of hours. In a typical experiment, a freshly prepared suspension gave 23 per cent of vegetative cells in 16 hours when the stock solutions were autoclaved on the preceding day, and 65 per cent when the stock solutions were autoclaved on the same day.

The number of vegetative cells may also be increased when the glucose, acetate solution is supplemented with as little as 1 part of potassium phosphate mixture in 2,000 parts of the solution. The effect is particularly noticeable when the stock solutions are not freshly autoclaved or when the spore suspension is relatively old. With a fresh suspension and stock solutions which were autoclaved 1 week previously, the proportion of vegetative cells obtained in 16 hours may be raised by the phosphate from about 12 to 20 per cent, with freshly autoclaved solutions and a suspension 23 days old, the proportion of vegetative cells may be raised from 8.9 to 28 per cent. Supplementing the glucose, acetate solution with Speakman's salts B or with biotin has no beneficial effect.

#### *Properties of the Vegetative Cells*

The vegetative cells that develop in the glucose, acetate solution have several interesting properties. In 70 to 90 per cent of these cells the cytoplasm becomes transparent to electrons at normal voltages and loses its property of staining with methylene blue at low pH (Knaysi and Baker, 1947). It also becomes gram negative, and the nuclei, which remain definitely gram-positive, may be demonstrated in these cells by gram staining. In the present work we used Burke's method modified by omitting the sodium bicarbonate. The electric charge of the surface of these cells and its relation to pH and cationic detergents are under

to those of cells of the same organism grown on ordinary media (Dyar and Knaysi, 1947). On the other hand, these cells seem to have undergone a considerable change in permeability. They are highly permeable to neutral red in extremely dilute solutions (e.g., 1 to 10 mg per liter).

Although the proportion of the spores which germinate in the glucose solution is, usually, relatively small, a high proportion of the spores which do not germinate acquire vegetative characteristics, becoming readily permeable to dyes in dilute solutions. Of these, many stain deeply and uniformly, but many others show a faintly stained cytoplasm containing two or more deeply stained nuclei, indicating loss of ribonucleic acid without germination. All gradations between these two extremes may be observed.

#### DISCUSSION

The present work confirms that of Knaysi (1945) and of Knaysi and Baker (1947) that the endospore of *Bacillus mycoides* contains a source of both nitrogen and phosphorus, but not of energy. Since microscopic observations reveal that the vegetative cells that develop in the glucose, acetate solution contain at least as much diffuse lipid material as those that develop in ordinary media, it is unlikely that the source of nitrogen and phosphorus would be a phospholipid. Since this source has a low isoelectric point (about pH 2), it is unlikely that it is protein. On the other hand, the low isoelectric point of this material is similar to, and its physiological behavior is identical with, that of volutin (Knaysi, 1946), which, according to Delaporte (1939), is chiefly ribonucleic acid. The present work further shows that when this material is used up, the cell becomes gram-negative, just as it becomes gram-negative when ribonucleic acid is removed by means of enzymes or chemicals (Dubos and MacLeod, 1938, Henry and Stacey, 1943). This conclusion is further confirmed by the work of Vendrely and Lipardy (1946), who attributed the poor stainability of cells hydrolyzed with hydrochloric acid to the loss of ribonucleic acid.

The present work also confirms that of Ruehle (1923) and others who demonstrated a number of enzymes, and that of Curran, Brunstetter, and Myers (1943), who demonstrated several minerals, in the endospores of various bacteria. Indeed, the endospore of *Bacillus mycoides* seems to be fully equipped to begin and carry on normal metabolic processes provided a source of energy is supplied. The coenzymes and minerals which may be necessary are probably held within the spore because of the low permeability of its coat. A spore which had lost its supply of coenzymes or minerals by leakage or in which these were somehow inactivated would not be able to germinate in the glucose, acetate solution. Analysis of the effect of aging of the spore suspensions indicates that aging is due to gradual oxidative processes that at first inactivate and later destroy something, in the spore, essential for metabolic activities, probably certain coenzymes. In the early stages, the process may be reversed by the addition of traces of phosphate or by the use of freshly autoclaved stock solutions, both heat and phosphate are known to activate solutions of sugar and of other complex biological material. The effect of autoclaving on dye-containing, complex culture media is common

knowledge among bacteriologists, the effect persists for hours. Dubos (1929) showed that autoclaved bouillon became more reducing and more suitable for the initiation of growth of small inocula. Wurmser (1930) reported that, below pH 9, the potential of sugar solutions is more positive when they are buffered with borate than when they are buffered with phosphate. Knaysi (1935) found that the potential of meat infusion was more quickly established and more definite and reproducible at 60 C than at room temperature, this potential was not affected by NaCl, but became more negative when phosphate was added at all pH values above 5.75. The beneficial effect of sublethal heat on spore germination observed by Evans and Curran (1943) and Curran and Evans (1945) appears very likely to be an effect of potential.

The present investigation does not favor the concept of dormancy as a significant biological phenomenon.

#### SUMMARY

The number of vegetative cells that develop in a solution of glucose and acetate inoculated with endospores of strain C<sub>2</sub> of *Bacillus mycoides* decreases with the age of the culture from which the spores were harvested and, particularly, with the age of the spore suspensions. When the spore suspension is moderately old, the number of vegetative cells may be considerably increased when the stock solutions of glucose and acetate are freshly autoclaved or when traces of potassium phosphate are added. When the spore suspension is too old, autoclaving and the addition of phosphate lose their effect. Aging is attributed to oxidative processes which, in the early stages, are reversed by the activated sugar.

The vegetative cells that develop in the glucose, acetate solution are gram-negative and highly permeable to neutral red. Many spores that do not germinate acquire vegetative characteristics.

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## SHIGELLA TIETE

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De Assis in 1939 (a,b) described strain 648, which was related in cultural properties to *Shigella alkaescens*, although it failed to ferment dulcitol. Antigenically it was entirely different from the ordinary *S. alkaescens*. At that time this form was called alkaescens II, a designation that was adopted by Neter (1944). Approximately 20 strains of this type were isolated from cases of dysentery in Rio de Janeiro and Sao Paulo (De Assis *et al*, 1946, De Assis, 1947b). Two of the strains slowly utilized lactose (De Assis *et al*, 1946). In a recent publication, De Assis (1947b) designated these strains as *Shigella tiete*<sup>1</sup>.

Among the strains employed by Braun and Unat (1942a,b, 1943a,b) in their investigation of the agglutinability of certain shigellae, a strain Clark was mentioned which, when received in this laboratory, proved to be culturally and serologically indistinguishable from *S. tiete*. The isolation of strains of this type at two points as distant as Brazil and Turkey suggested that *S. tiete* is more than a local Brazilian variant. For this reason, and because the confusion regarding this type reflects what may occur in the classification of shigellae in general, we feel justified in reporting our experiences with *S. tiete*.

The strain Clark was obtained through the courtesy of Professor Braun both as "Ocl" and "Ol" variant. Dr. de Assis kindly sent us four of his strains including the lactose-fermenting cultures, "Gen" and "Cav". We are also indebted to Drs. Ewing, Francis, Neter, and Mollan for strains used in this work.

*S. tiete* is a gram-negative rod that is nonmotile in fluid media and semisolid agar at 20 C and 37 C. It does not liquefy gelatin nor attack urea. It is anaerogenic, indole-positive, and it reduces dimethylamine oxide (Wood and Baird, 1943, Weil and Black, 1944). It forms acid rapidly from glucose, mannitol, maltose, and arabinose, and may or may not slowly utilize salicin. Dulcitol and rhamnose are not fermented. Lactose was not acidified by two of the strains of Dr. de Assis, but was slowly fermented by strains "Gen" and "Cav" as well as by Braun's strain Clark. On agar plates, the strains form both clear and opaque colonies, as observed in *S. alkaescens* (see figure 1 and references in Weil, 1947). All strains formed round smooth colonies. They grow evenly throughout fluid media, and suspensions are salt-stable.

The clear variant is agglutinable. The opaque variant is agglutinable when tested in the living state, but after boiling for 1 hour the bacteria are completely agglutinable. The clear variant usually breeds true. The opaque form has a tendency to split off clear variants. The inclination to do so varies greatly in the progeny of individual colonies picked and repeatedly replated.

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<sup>1</sup> Pronounced tee ay-tay.

When tested by slide agglutination with absorbed sera or in tubes with unabsorbed sera for *Shigella dysenteriae*, *Shigella ambigua*, the 15 types of Flexner (I to XIV and *Shigella elousae*), *Shigella sonnei*, *S. alkalescens*, and the 5 types of the Large Sachs group, no agglutination was obtained. Also no significant reactions were obtained with living or boiled bacteria with sera for *Shigella uakelield*, strains 1831 and 2370 of Wheeler, Neter's 9731 (*alkalescens* type IV), and a few hitherto undescribed single strains, including the culturally similar strain A113, which Dr. J. B. Nelson isolated in India as no. 1477-C857. Boiled bacteria reacted to approximately 10 per cent of the homologous titer with sera for *Shigella rio* (Weil *et al.*, 1948), Boyd's strain 1296/7, and Ewing's 2-193. In all three cases absorptive analysis showed that minor and independent anti-



FIG. 1. TWENTY-FOUR-HOUR CULTURE ON AGAR SHOWING THE "CLEAR" AND THE "OPAQUE" VARIANTS OF A STRAIN OF *SHIGELLA FLEXNERI*. MAGNIFICATION APPROXIMATELY 2X.

genic relations are involved. As to *S. rio*, there was no evidence that absorption with one of the *S. flexneri* strains influenced significantly the phase B antigen.

When rabbits were immunized with *S. flexneri* strains, sera were obtained that gave identical reactions when tested against the different *Shigella* types listed in the previous paragraph. They could be exhausted by each of the *S. flexneri* strains. It made no difference whether live or boiled cultures were used. Absorption of the sera made with living microorganisms by bacteria boiled for 1 or 2 hours also completely exhausted the sera.

Significant cross reactions were observed with strains of Ewing's 2-193 and with Boyd's 1296/7. Absorption with these strains showed that the homologous reaction is not significantly impaired and the antibodies reactive with the two strains are not identical. Strain 9731 of Neter also showed some cross reaction. Absorptive analysis showed that this antigen is related to the one present in Boyd's 1296/7.

## DISCUSSION

Our data are in complete agreement with those of De Assis. *S. tiele* appears to be a distinct new member of the genus *Shigella*. It shares with other shigellae (see references in Weil, 1947) the property of a heat-labile component or structure that inhibits agglutination in the living state. Variants are formed (and possibly also may occur in natural surroundings) in which this "envelope" is lacking. The agglutinable and inagglutinable variants can be distinguished by differences in the opacity of their colonies, which is another character that *S. tiele* shares with *S. alkalescens*.

It appears that otherwise indistinguishable strains of *S. tiele* may differ in their ability to ferment lactose slowly. In a recent review (Weil, 1947), occasion was taken to emphasize the variability of the cultural properties of *Shigella* types. Thus, *Shigella paradysenteriae* types IV and VI comprise strains that may or may not utilize mannitol, and Flexner type VI also comprises strains that may or may not form slight amounts of gas (references in Weil, 1943, 1947). From the general agreement on these cases it appears that the viewpoint that antigenic relationships are of greater importance than cultural properties for defining *Shigella* types is gaining wider acceptance. This attitude will probably facilitate the correlation of classification and immunological host relations (Weil, 1947).

In the case of *S. tiele* the range of variation extends to a property that in the past appeared to be of particular importance in classification, namely, to the utilization of lactose. However, *S. tiele* is not the only *Shigella* type in which lactose fermentation varies. The antigenic characteristics of the *Shigella* (2-193) that was first found by Ewing, and which is identical with Neter's *alkalescens* III (Neter, 1944, De Assis, 1947a), are found in strains that do and that do not form acid from lactose (Carpenter, 1943, Carpenter and Stuart, 1946). Under the predominance of cultural considerations, Ewing's 2-193 has been successively classified as a *S. alkalescens*, as *S. dispar* (Carpenter and Stuart, 1946), and as *S. flexneri* (Francis, 1946). Similar uncertainties have been avoided in the description of salmonellae by adopting the practice of designating new types by some geographical epithet. It may be advisable to follow a similar procedure in the designation of new shigellae. Accordingly, a Brazilian *Shigella* has recently been described by Weil *et al.* (1948) as *Shigella rio*, and this consideration induced De Assis to abandon his former designation of *S. alkalescens* II in favor of *S. tiele*.

## SUMMARY

Data on the cultural and antigenic properties of *Shigella tiele* are given.

The reasons for favoring the designation of new shigellae by geographical epithets are discussed.

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## NOTES

### NUTRITIONAL REQUIREMENTS OF *BACILLUS LARVAE*<sup>1</sup>

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*Bacillus larvae*, causal agent of American foulbrood of bees, is a fastidious organism requiring special media for successful cultivation. Crushed blood, egg yolk, yeast, carrot and turnip extracts, and chicken embryo have been used for this purpose, but no attempt to elucidate the specific nutritional requirements of this organism was made until 1942, when it was found to require thiamine for growth on an agar medium containing peptone, glucose, and inorganic salts (Lochhead J Bact, 44, 185). The peptone could not be replaced by casein hydrolyzate or a mixture of known amino acids. An attempt to develop a completely synthetic medium for this organism is briefly reported herein.

Ten growth factors and 18 amino acids (Katznelson and Lochhead J Bact, 54, 83) were added in different combinations to a basal salts glucose fluid medium, and the mixtures inoculated with heavy suspensions of *B. larvae* cells from a 48-hour peptone yeast extract glucose agar culture (Holst and Sturtevant J Bact, 40, 723). Although only fair growth was obtained in this medium, the requirement for thiamine was confirmed with all strains tested. The thiazole and pyrimidine fractions of this vitamin could not replace the intact molecule. None of the remaining growth factors was required, however, subsequent tests with streptogenin<sup>2</sup> indicated that it was essential for some, though not for all, strains of *B. larvae*. A purine base was necessary for growth of all strains, xanthine and guanine being interchangeable, though adenine was somewhat less effective.

Omission of each amino acid singly from the mixture of 18 in a basal medium containing a purine base in addition to the original ingredients showed that histidine was essential for the growth of all strains. In the absence of cystine, valine, leucine, aspartic acid, tryptophan, arginine, proline, or isoleucine growth was retarded. Considerable strain variation was observed in regard to requirements for these amino acids. The nine acids mentioned above supported fair growth of 12 strains. However, serial transfer was not achieved even in a fluid medium containing 18 amino acids. This was accomplished only on the addition of washed agar to give either a semisolid or solid medium.

Slightly different results were obtained in regard to amino acid requirements when a semisolid agar instead of a fluid medium was used. The omission of histidine or proline suppressed growth completely, as did the omission of tyrosine.

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<sup>2</sup>Kindly supplied by Dr. D. W. Woolley, Rockefeller Institute for Medical Research, New York.

and phenylalanine together, though not singly, without arginine growth was very slight. The remaining amino acids found to be stimulatory in a fluid medium were omitted without effect from the semisolid agar medium.

The need for heavy inocula to initiate growth in both fluid and semisolid media and our inability to culture the organism serially in the liquid medium suggest additional growth essentials. This is borne out by the fact that washed cells grew better in a fluid peptone medium than in the synthetic medium and by the ability of the organism to grow on serial transfer in the former. The replacement of agar by other substances providing increased surface, such as glass wool, crushed glass, and glass beads, was ineffective in stimulating growth, as was aeration.

## MIMA POLYMORPHA IN MENINGITIS

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A perusal of the bacteriological literature shows that the bacteria isolated from the spinal fluid in meningitis in sporadic cases may be classified into three groups: (1) a very high percentage of the pathogenic bacteria, (2) many bacteria that are not ordinarily considered pathogenic, and (3) new species that may or may not bear a resemblance to *Neisseria* or other known bacteria. Within the genus *Neisseria*, smear preparations are not sufficient for identification of the microscopically similar species. Murray (Urol and Cutaneous Rev, 43, 739) called attention to the importance of the cultural identification with respect to the meningococcus and the gonococcus and was confirmed later by Carpenter and Charles (Am J Pub Health, 32, 640). Branham (U S Pub Health Service, Pub Health Repts, 45, 845) discovered *Neisseria flavescens* by the cultural method during a localized epidemic of meningitis. Pleomorphism is another factor that can confuse a diagnosis by smear preparations. This has been shown by the author (J Bact, 38, 119, Iowa State Coll J Sci, 16, 171, J Lab Clin Med, 28, 710).

A patient (J C) was admitted to the hospital in a comatose condition. In the routine examination of the spinal fluid a gram-negative intracellular diplococcus was isolated that resembled the genus *Neisseria*. Since it was obtained from the spinal fluid, it was assumed for the moment to be *Neisseria intracellularis*. However, the cultural characteristics did not conform to that organism and it was given by Miss Margaret Bush to the author for identification. Further studies showed the organism to be *Mima polymorpha*. Cultures from the spinal fluid yielded this organism during the first week of illness, and during this time micro-

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scopic examinations of the spinal fluid continued to show gram-negative diplococci. After the first week, when treatment with sulfonamides had begun, further smears and cultures were negative. This continued to be the case to the time of the patient's discharge from the hospital 10 weeks later. When the patient was last seen a year and a half after his discharge from the hospital, he reported that he had been in excellent health since leaving the hospital.

This culture of *Mima polymorpha* and other members of the tribe *Mimeae* were pathogenic to mice when injected intraperitoneally. Injections were made late in the afternoon and the mice were found dead the next morning. Cultures were taken from the heart blood. Larger doses were used in this work than those used by Deacon (J Bact, 49, 511) with guinea pigs. Since the rod form predominates after culture on media for some time, the mouse inoculation method was used to revert the *Mimeae* cultures to the condition in which the majority were diplococcal forms.

Regardless of the source of real or apparent gram-negative diplococci, whether eye, cervix, vagina, spinal fluid, or brain tissue (Deacon), cultural studies must be made for a correct identification of the bacteria.

## THE EFFECT OF STREPTOMYCIN ON THE FORMATION OF ADAPTIVE ENZYMES

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Certain mycobacteria oxidize benzoic acid, and this oxidation is inhibited by streptomycin (Fitzgerald and Bernheim J Bact, 54, 671). Organisms grown on a normal medium exhibit a latent period of 30 to 60 minutes before benzoic acid is oxidized. Organisms grown in benzoic acid subsequently oxidize benzoic acid without this latent period, and much more streptomycin is necessary to inhibit the oxidation. This relative insensitivity to streptomycin was thought to be due to the presence of more benzoic acid oxidase in the organisms grown in, and therefore adapted to, benzoic acid. Recently Stanier (J Bact, 54, 339) has shown that benzoic acid and related compounds are oxidized by adaptive enzymes in *Pseudomonas fluorescens*. The adaptation occurs when the compounds are added to nongrowing cell suspensions in the Warburg vessel, and it requires about an hour for the formation of the adaptive enzyme. It seems possible that a similar rapid adaptation occurs in mycobacteria. If this is so, then the inhibition of benzoic acid oxidation by streptomycin may not be due primarily to the inhibition of the benzoic acid oxidase but to the inhibition of the formation of the enzyme. In other words, streptomycin may inhibit the production of an adaptive enzyme.

In order to test this, *Mycobacterium lacticola* was grown in a medium already described. Forty mg per cent neutralized benzoic acid was added to the complete medium. After 48 hours' growth at room temperature the bacteria were centrifuged and washed, and a standard suspension was made. Aliquots were placed in Warburg vessels, and the oxygen uptake was measured in M/20 phosphate buffer pH 6.7 in the presence of benzoic acid with and without different amounts of streptomycin. Table 1 shows that the oxidation of benzoic acid by organisms grown in the normal medium is inhibited 44 per cent by 50  $\mu$ g per ml, 82 per cent by 100  $\mu$ g per ml, and completely by 200  $\mu$ g per ml of strepto-

TABLE 1

*The effect of streptomycin on the oxidation of 1.0 mg sodium benzoate by M. lacticola at 37°C pH 6.7*

TIME Min	NORMAL				GROWN IN 40 MG % BENZOATE			
	Control	50 $\mu$ g S	100 $\mu$ g S	200 $\mu$ g S	Control	50 $\mu$ g S	100 $\mu$ g S	200 $\mu$ g S
30	-6	-10	-4	-6	93	71	64	66
60	4	-2	-4	-8	276	216	195	185
90	59	28	9	-9	427	332	301	279
120	139	73	25	-6	554	449	408	379
150	229	119	40	-7	563	526	478	441

The oxygen uptakes in the absence of benzoate have been subtracted. The figures are in terms of mm<sup>3</sup> of oxygen utilized. S = streptomycin. The minus sign indicates an inhibition of the resting respiration by the benzoate.

mycin. On the other hand, the oxidation of benzoic acid by organisms grown in its presence is inhibited 25 per cent by 50  $\mu$ g per ml, 30 per cent by 100  $\mu$ g per ml, and 33 per cent by 200  $\mu$ g per ml. Similar results are obtained when *p*-hydroxybenzoic acid is used. These experiments suggest that the enzymes are relatively insensitive to streptomycin and that inhibition is caused by the suppression of their formation. The previously published experiment that showed that streptomycin caused less inhibition in the presence of 2.0 mg of benzoic acid than in the presence of 1.0 mg could be interpreted to mean that 2.0 mg caused the production of more enzyme.

## STERIOD EFFECT UPON BACTERIAL GROWTH

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Raab (Science, 103, 670) recently reported that cholesterol added to culture media inhibited the growth of *Staphylococcus aureus*. In this laboratory, 0.1 per cent cholesterol in nutrient broth either inhibited or enhanced the growth of cer-



tain gram-negative organisms but was without effect upon the gram-positive bacteria tested

TABLE 1  
Growth of bacteria in nutrient broth containing steroids\*

	BICHO- LESTERYL	CHOLES- TERYL CHLORIDE	CHOLES TERYL BROMIDE	CHOLES TEROL	CHOLES TERYL p TOLU ENE SUL- FONATE	5-CHO- LESTENE	3 5-CHO LESTA DIENE	CONTROL
<i>Alcaligenes faecalis</i>	N	N	N	E	N	N	N	N
<i>Staphylococcus aureus</i>	N	N	E	N	N	N	N	N
<i>Aerobacter aerogenes</i>	N	E	N	I	N	N	E	N
<i>Escherichia coli</i>	N	E	E	N	N	N	I	N
<i>Proteus vulgaris</i>	N	N	N	N	N	N	N	N
<i>Eberthella typhosa</i>	N	E	N	N	N	N	N	N
<i>Shigella paradysenteriae</i>	N	E	N	I	N	N	N	N
<i>Bacillus subtilis</i>	N	N	N	N	N	N	E	N
<i>Salmonella schottmuelleri</i>	N	E	E	E	N	N	N	N
<i>Staphylococcus albus</i>	N	N	N	N	N	N	E	N
<i>Klebsiella pneumoniae</i>	N	I	I	I	N	N	E	N
<i>Streptococcus lactis</i>	N	I	I	N	I	I	N	N
<i>Bacillus megatherium</i>	N	N	N	N	N	N	N	N
<i>Serratia marcescens</i>	N	N	N	N	N	N	N	N
<i>Sarcina lutea</i>	N	N	N	N	N	N	N	N

N = normal growth

E = enhanced growth

I = inhibited growth

\* Nutrient broth contained 0.1 per cent steroid derivative. Readings were measured by turbidity.

The steroid derivatives, which were prepared by replacing the hydroxy group of cholesterol with halogen or other substituents, generally affect the growth of bacteria differently. This is shown in table 1.

The action of cholesteryl chloride and bromide is particularly interesting in view of the fact that Turfitt (J. Bact., 54, 557) found many soil organisms unable to utilize cholesteryl chloride.



# AMINO ACID INTERRELATIONSHIPS IN THE NUTRITION OF *STREPTOCOCCUS BOVIS*

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During the course of an investigation to determine the nutritive requirements of *Streptococcus bovis*, a variable growth response was noted with certain combinations of amino acids that could not later be explained on the basis of the amino acid requirements determined for that organism. *Streptococcus bovis* was found to be unusual among the streptococci in that it does not have an absolute requirement for any single amino acid. Moreover, it can initiate growth in a medium containing one amino acid, arginine (Niven, Washburn, and White, 1948). It was found, however, that the addition of certain other amino acids or combinations of amino acids to a basal medium containing arginine produced growth retardation or inhibition. This growth inhibition can be reversed by the addition of certain other amino acids.

The qualitative and quantitative interrelationship between these amino acids is the subject of the present study.

## METHODS

The culture used throughout this study was *Streptococcus bovis* (P20), from a fatal case of subacute bacterial endocarditis. The composition of the basal medium is given in table 1. Five mg of arginine per 10 ml of medium supply sufficient amino nitrogen for maximum growth of the organism. The concentration of sodium thioglycolate was increased to 5 mg per 10 ml to ensure an adequate sulfur source. The following amino acids were used during the investigation: DL-threonine, DL-methionine, DL-valine, DL-leucine, DL-isoleucine, DL-lysine, L-histidine, DL-phenylalanine, L-tyrosine, L-proline, glycine, DL-alanine, DL-serine, L-glutamic acid, L-aspartic acid, L-cystine, L-tryptophan, DL-norleucine, and L-hydroxyproline.

The technique used in this study including the methods of treating glassware, the preparation of the medium, incubation, and the technique for measuring growth were previously described (Niven, Washburn, and White, 1948). The only change was in the method used for inoculating the medium. The cells in a 24-hour meat infusion broth culture were centrifuged and resuspended in 1 ml of sterile water. One drop, or approximately 0.05 ml of this cell suspension, was mixed with 9 ml of sterile distilled water. The medium under test was inoculated with one drop of this dilute cell suspension.

## RESULTS

By adding the 19 amino acids individually to the basal medium in varying concentrations, it was found that certain of these amino acids produced either

growth retardation or inhibition depending upon the concentration. As shown in table 2, isoleucine, leucine, threonine, norleucine, and alanine produced growth inhibition when added individually to the medium. The minimum concentra-

TABLE 1  
*Basal medium*

	<i>micrograms</i>
Riboflavin	10
Calcium pantothenate	10
Nicotinic acid	50
Pyridoxine	10
Thiamine HCl	10
Biotin	0.01
Folic acid (concentrate)	0.01
p-Aminobenzoic acid	10
Choline chloride	50
Inositol	50
Xanthine	50
Adenine sulfate	50
Guanine HCl	50
Uracil	50
	<i>milligrams</i>
L-Arginine	5
Glucose	100
K <sub>2</sub> HPO <sub>4</sub>	60
Sodium thioglycolate	5
Salts*	
Water to make	10 ml

\* Salts composed of 20 mg NaCl, 0.8 mg MgSO<sub>4</sub> · 7H<sub>2</sub>O, 40 micrograms FeSO<sub>4</sub> · 7H<sub>2</sub>O, and 12 micrograms MnCl<sub>2</sub> per 10 ml medium.

TABLE 2

*Amino acids producing growth inhibition in the presence of L arginine (5 mg per 10 ml)*

INHIBITOR	MINIMUM CONCENTRATION FOR COMPLETE INHIBITION
	<i>mg per 10 ml</i>
DL-Isoleucine	0.002
DL-Leucine	0.01
DL-Threonine	0.2
DL-Norleucine	0.5
DL-Alanine	5.0
DL-Phenylalanine	
+	
L-Tyrosine	0.2 each.

tion of each of these that inhibited growth in the medium containing 5 mg of arginine varied from 0.002 mg per 10 ml for isoleucine to 5 mg per 10 ml for alanine. It has been pointed out by Hegsted and Wardwell (1944) that many

samples of synthetic DL-leucine contain significant amounts of isoleucine. The leucine used in this study was isoleucine-free.

A combination of phenylalanine and tyrosine was found to inhibit growth, whereas either of these amino acids when added separately in as high a concentration as 20 mg per 10 ml produced no discernible effect upon the growth of this organism. As low a concentration as 0.2 mg per 10 ml of each of these amino acids completely prevented growth. By increasing or decreasing the concentration of one of these amino acids and holding the other constant it was found that

TABLE 3

*Quantitative relationship between DL  $\beta$ -phenylalanine and L-tyrosine in growth inhibition*

DL-PHENYLALANINE	L TYROSINE	DENSITOMETER READINGS*
<i>mg per 10 ml</i>	<i>mg per 10 ml</i>	
5	1	31
2	1	0
1	1	0
0.5	1	0
0.2	1	100
0.1	1	150
0	1	155
1	5	61
1	2	0
1	1	0
1	0.5	0
1	0.2	80
1	0.1	130
1	0	125
5	5	0
2	2	0
1	1	0
0.5	0.5	0
0.2	0.2	0
0.1	0.1	120
0	0	145

\* Readings made after 47 hours' incubation, uninoculated medium reads zero

the greatest inhibitory effect occurs when equal concentrations of phenylalanine and tyrosine are present (table 3).

As would be expected, the amount of arginine in the medium affects the concentration of the inhibitory amino acids needed to prevent growth. By doubling the arginine in the medium it was found that a substantially larger amount of the antagonistic amino acid was needed.

The amino acids that were found to reverse the inhibition produced by isoleucine, leucine, threonine, and norleucine are given in table 4. In each case valine was an effective counteracting substance. It produced complete reversal at a concentration lower than that of the inhibiting agent. Glutamic acid was the next most effective amino acid, whereas cystine and methionine were usually

TABLE 4

*Relationship between the inhibitory amino acids and those that counteract their inhibition*

INHIBITING AMINO ACID AND CONCENTRATION	COUNTERACTING AMINO ACIDS	MINIMUM CONCENTRATION
		<i>mg per 10 ml</i>
DL-Isoleucine 0.2 mg per 10 ml	DL-Valine L-Glutamic acid DL-Leucine DL-Methionine L-Cystine	0.05 0.2 1.0* 5 5
DL-Leucine 1 mg per 10 ml	DL-Valine L-Glutamic acid DL-Isoleucine L-Cystine DL-Methionine	0.01 0.05 0.2* 0.5 1
DL-Threonine 1 mg per 10 ml	DL-Valine L-Glutamic acid DL-Methionine L-Cystine	0.05 0.5 0.5 5
DL-Norleucine 2 mg per 10 ml	DL-Valine L-Glutamic acid DL-Methionine L-Cystine	0.05 0.5 0.5 5

\* Maximum, not minimum, concentration

TABLE 5

*Effect of tryptophan upon growth inhibition produced by phenylalanine and tyrosine*

DL-PHENYLALANINE	L TYROSINE	L-TRYPTOPHAN	DENSITOMETER READINGS*
<i>mg per 10 ml</i>	<i>mg per 10 ml</i>	<i>mg per 10 ml</i>	
1	1	1	195
1	1	0.5	190
1	1	0.1	190
1	1	0.05	190
1	1	0.01	87
1	1	0.005	57
1	1	0.001	57
1	1	0.0005	89
1	1	0.0001	16
1	1	0	0
0	0	1	190
0	0	0	190

\* Readings made after 40 hours' incubation, uninoculated medium reads zero

effective only at concentrations equal to or higher than that of the inhibiting amino acid

Leucine, which alone produced growth inhibition, was also found to counteract isoleucine. In this case, not high but only low concentrations were effective. As would be expected, low concentrations of isoleucine were also found to counteract the inhibition produced by leucine.

The growth inhibition produced by the simultaneous addition of phenylalanine and tyrosine was found to be counteracted by tryptophan. As shown in table 5, as low a concentration as 0.05 mg of tryptophan per 10 ml would completely reverse the growth inhibition produced by 1 mg each of phenylalanine and tyrosine. Partial counteraction was obtained with as little as 0.0001 mg of tryptophan. In addition, it was found that both indole and anthranilic acid were effective substitutes for tryptophan in overcoming this growth retardation. Counteraction can also be obtained using glutamic acid or cystine in concentrations equal to or higher than that of the phenylalanine and tyrosine.

#### DISCUSSION

It would appear that studies of amino acid interrelationships which manifest themselves by inhibiting the growth of an organism in a simple, chemically defined medium might divulge helpful information as to possible paths of amino acid syntheses by microorganisms. One of the earliest reports of antagonistic action of amino acids upon the growth of bacteria was presented by Gladstone (1939) using *Bacillus anthracis*. In this case, interrelationships were shown to exist between valine, leucine, and isoleucine, norleucine, valine, and leucine, and valine and threonine. The mechanisms observed in the present study may be very similar to those reported by Gladstone. However, it was necessary to use a number of amino acids in the basal medium for the growth of *Bacillus anthracis*, and this may be the reason that the amino acid interrelationships manifest themselves in a somewhat different manner from those in the present study.

In more recent years amino acid interrelationships have been demonstrated by a number of authors using a variety of microorganisms. Porter and Meyers (1945) have shown that certain concentrations of allothreonine, norleucine, and norvaline inhibit the growth of *Proteus morganii* when added singly to a medium containing cystine. A number of amino acids were found that would counteract the growth inhibition produced by allothreonine and norvaline, whereas methionine was the only amino acid that would counteract the effect of norleucine.

Mutants of *Neurospora* have also been of value in the study of the metabolism of amino acids. Bonner (1946), using a strain of *Neurospora* that requires both valine and isoleucine, has proposed that a genetic block in the conversion of ketoisoleucine to isoleucine operates in this organism to bring about an accumulation of ketoisoleucine. This in turn may result in an inhibition of valine synthesis.

In the present study, growth inhibition was observed in the basal medium with added isoleucine, leucine, threonine, or norleucine. Since the growth inhibition could be reversed using relatively small amounts of valine in each case, it may be supposed that each of these amino acids is effectively blocking the synthetic processes at the valine level.

A study of the minimum concentrations of each of the inhibiting amino acids that will completely prevent growth of the organism demonstrates the fact that isoleucine is by far the most effective. Leucine, threonine, and norleucine must be added in progressively larger amounts to produce complete inhibition of growth. This might lead one to assume that the only reaction blocked in this case is the synthesis of valine in the presence of too high a concentration of isoleucine. The presence of added leucine, threonine, or norleucine may produce the same effect owing to their structural similarity. Another possibility lies in the fact that added leucine, threonine, or norleucine may bring about the synthesis of more isoleucine.

In each case considerably higher concentrations of glutamic acid, methionine, and cystine were also effective in reversing the inhibition produced by isoleucine, leucine, threonine, and norleucine. Here we may consider the possibility that they bring about the reversal indirectly through the ability of the organism to synthesize valine and other amino acids from them. These points are admittedly mere suppositions with little foundation.

Hier (1947) noted that when relatively large amounts of leucine, isoleucine, or methionine were given by *gavage* to dogs, a significant decrease in the level of certain free amino acids in the plasma accompanied the rise in the plasma level of the amino acid fed. The amino acids affected in this way varied with the particular one administered, however, it should be noted that in each case the plasma level of valine was significantly decreased. Whether these observations have any bearing upon the present work is unknown.

In competitive inhibition studies using analogues of phenylalanine, Beerstecher and Shive (1946) have shown that tryptophan is highly effective in reversing the toxic manifestations produced by  $\beta$ -hydroxyphenylalanine and  $\beta$ -2-thienylalanine. The results of this work indicate that the tryptophan was transformed to phenylalanine and thus reversed the toxicity of the analogues.

In addition, Beerstecher and Shive (1947) have shown that a very high concentration of tryptophan in a medium in comparison to the amount of phenylalanine present will inhibit the growth of *Streptococcus faecalis* R as well as certain other organisms. It was concluded in this case that tryptophan was competing with phenylalanine for a site in an essential enzyme system.

In the present study it was found that a combination of DL-phenylalanine and L-tyrosine prevented the growth of *Streptococcus bovis* in the medium used. Equal concentrations of each of these amino acids were most effective, whereas singly they had no effect on the growth of the organism. Rose and Womack (1946) have shown that both of the optical isomers of phenylalanine are utilized in the rat. No attempt was made to determine the effect of the unnatural isomer upon *Streptococcus bovis*.

A relatively small amount of tryptophan is needed to reverse the inhibition produced by phenylalanine and tyrosine. Snell (1943) showed that *Lactobacillus arabinosus* is capable of utilizing indole and anthranilic acid as a substitute for tryptophan. In the present study it was found that both indole and anthranilic acid will effectively reverse the growth inhibition produced by phenylalanine and



tyrosine It would be interesting to speculate that phenylalanine and tyrosine may prevent the growth of *Streptococcus bovis* by blocking the synthesis of tryptophan However, it is very difficult to visualize the necessity for both of these amino acids in approximately equal concentrations to produce this effect

Although the foregoing experiments are incomplete and preliminary in nature, they would seem to contribute, at least in small part, to the increasing literature on the subject of amino acid antagonisms, which exist among both microorganisms and animals These accumulating data offer material for rather interesting speculations as to the mode of some of the little-known synthetic mechanisms

All cultures of the particular variety of *Streptococcus bovis* used in this study were identical with respect to these amino acid antagonisms However, it was impossible to demonstrate these phenomena with the other species of streptococci tested, perhaps because of their more exacting nutritional requirements It would therefore appear that these strains of *Streptococcus bovis* might be valuable microorganisms for the study of some of the synthetic mechanisms with respect to the amino acids

#### SUMMARY

Small amounts of isoleucine and larger amounts of leucine, threonine, and norleucine inhibit the growth of certain strains of *Streptococcus bovis* in a basal medium containing one amino acid, arginine The inhibition produced by each of these amino acids can be reversed by the addition of small amounts of valine and larger amounts of glutamic acid, methionine, and cystine, respectively

Equal amounts of DL-phenylalanine and L-tyrosine inhibit the growth of the organism, although they produce no effect when added singly This inhibition can be reversed by using small amounts of tryptophan and larger amounts of glutamic acid or cystine

A possible explanation of the inhibition produced by isoleucine, leucine, threonine, and norleucine is that they block the synthesis of valine by the organism, whereas tyrosine and phenylalanine may block the synthesis of tryptophan and thus prevent the multiplication of the organism

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# THE ACTION OF BACILLUS CEREUS AND RELATED SPECIES ON THE LECITHIN COMPLEX OF EGG YOLK

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A bacterial spoilage of some fertile eggs used in the cultivation of mouse mammary tumor produced an unusual reaction in the eggs' constituents. The yolks of these eggs were hardened, had the appearance of ping-pong balls, were creamy in color, and upon standing in a container would crack open indicating their marked change in consistency. Their albumen was tan in color, and it and the yolk had a sour, cheesy odor. The organism causing these changes was found to be a member of the aerobic mesophilic sporeforming group, and was identified as *Bacillus cereus*.

Since the changes brought about in the eggs by the bacterium were so unusual and since the inoculated eggs did not possess those odors commonly associated with many bacterial spoilages, it was thought to be of interest to determine what substance or substances in the egg were involved in the chemical and physical alterations and also to determine whether other members of this group of organisms possessed similar powers.

## EXPERIMENTAL RESULTS

Preliminary tests with the sporeformer on the albumen and yolk of aseptically opened fresh eggs indicated that the action that brought about the changes in the whole egg was centered in the yolk. Since Winton and Winton (1937) indicate that water (48.8 per cent), glycerides (18 per cent), protein (16 per cent), and phospholipids (10 per cent) are the major ingredients in egg yolk, tests were made for the detection of a lipase from the organism that might be capable of acting on the yolk fat. The tests were negative. Tests made on yolk protein extracted from fresh eggs showed a rapid proteolysis of it by the organism, but there was no indication that the physical alterations of the yolk in the inoculated whole egg could be due to this type of action.

MacFarlane, Oakley, and Anderson (1941), van Heyningen (1941), and Weed, Minton, and Carter (1942) worked with a lecitho-vitellin suspension in their study of the  $\alpha$ -toxin of *Clostridium welchii*, and found that this toxin altered the suspension and produced a characteristic flocculation. McClung, Heidenrich, and Toabe (1946) in their work with members of the genus *Clostridium* suggested a substitute egg yolk agar medium for that used by Hayward (1943) and Nagler (1945). In this medium a precipitate formed in the agar following the growth of some of the organisms. These findings suggested that the phospholipid fraction of the yolk might be concerned in the alterations noted in the spoiled eggs.

To determine whether the isolated organism had any action upon the phospholipid component, the McClung, Heidenrich, and Toabe (1946) egg yolk medium

was used as the solid substrate with the substitution of 10 grams of tryptone for the 40 grams of proteose peptone no 2. The lecitho-vitellin suspension of van Heyningen (1941), dispensed in sterile 12-by-100-mm test tubes, was used as the liquid medium. Cells from an agar slant were used for inoculation. Incubation was at 37 C for 48 to 72 hours.

The isolated organism acted upon the lecitho-vitellin suspension to produce, at first, an opalescence that upon continued incubation became flocculent and then later appeared as a curd floating upon a clear liquid. On the egg yolk medium there was a rapid formation of a creamy precipitate extending below and out from the spot of inoculation. These two types of action indicated that the isolate in its growth in the whole egg was probably decomposing the phospholipid component of the yolk. Since lecithin has been reported (Winton and Winton, 1937) to be in a larger amount than cephalin in the phospholipid fraction of egg yolk, and since MacFarlane (1942) concluded that the  $\alpha$ -toxin of *C. welchii* does not attack either the amino-ethanol or the serine form of cephalin, a test of the action of the organism on lecithin was thought to be advisable.

Lecithin was prepared according to the methods of MacFarlane and Knight (1941) and Levene and Rolf (1927). The former method does not free the lecithin from cephalin and sphingomyelin, whereas the latter does. The method for putting the lecithins in suspension was that of King (1931). One volume of a 2.5 per cent alcoholic solution of a lecithin was run into 10 volumes of hot borate buffer at pH 7.5 with vigorous shaking. This gave a stable emulsion.

The isolate produced an opalescence in both lecithin suspensions similar to the reaction in the van Heyningen (1941) medium, however, there was less reaction in the lecithin suspensions than that normally found in the lecitho-vitellin preparation. It was noted that a cell-free filtrate, secured by Seitz-filtering the liquid portions of eggs inoculated with the isolate, acted readily upon either the egg yolk medium or upon the lecithin-borate suspension. Figure 4 shows the action of this filtrate upon the lecithin-borate suspension.

When the whole egg was used as the test medium, it was thoroughly washed, the air sac end was treated with 1:100 HgCl<sub>2</sub>, a small pore was drilled into the treated area with the heated end of a metal file, and then 1 ml of a saline suspension of cells from a 24-hour-old agar slant was injected into the yolk by means of a 5-ml syringe equipped with an 18-gauge needle 1½ inches long. Immediately after the inoculation was made, the pore was closed with sterile paraffin. A control egg was used that had received 1 ml of sterile saline. Incubation was at 37 C for 48 to 72 hours.

To determine how widespread the possession of lecithinase might be within the genus *Bacillus*, other species were tested for their ability to form a precipitate on the egg yolk agar medium, to form an opalescence in the lecitho-vitellin suspension, and to harden the yolk in a whole egg. Since Smith, Gordon, and Clark (1946) have proposed certain modifications in the grouping of the aerobic mesophilic sporeforming bacteria, and particularly for the relation of *Bacillus mycoides*, *Bacillus anthracis*, *Bacillus praussnitzii*, and *Bacillus albolactis* to *Bacillus cereus*, cultures of these were especially noted. The results of these tests are given in table 1.

TABLE 1  
*Reactions of some bacilli on lecithin containing media*

CULTURES USED	HARDENING OF YOLK IN WHOLE EGG	PRECIPITATE IN EGG YOLK AGAR	FLOCCULATION IN LECITHIN VITELLIN SUSPENSION
Departmental cultures			
Isolate	++++	+++	+++
<i>B. cereus</i>	+++	++	+++
<i>B. mycoides</i>	++	++	+
<i>B. subtilis</i>	—	—	—
<i>B. mesentericus</i>	—	—	—
<i>B. circulans</i>	—	—	—
<i>B. megatherium</i>	—	—	—
3 unidentified bacilli	—	—	—
N R Smith cultures			
<i>B. cereus</i>	+++	++	++
<i>B. cereus</i> var. <i>mycoides</i>	++	++	++
<i>B. megatherium</i>	—	—	—
<i>B. pumilus</i>	—	—	—
<i>B. subtilis</i>	—	—	—
<i>B. alvei</i>	—	—	—
<i>B. circulans</i>	—	—	—
<i>B. brevis</i>	—	—	—
<i>B. macerans</i>	—	—	—
<i>B. polymyxa</i>	—	—	—
Kenneth Burdon cultures			
<i>B. cereus</i>	+++	++	++
<i>B. mycoides</i>	+++	++	+
<i>B. anthracis</i>	+	+	±
<i>B. sphaericus</i>	—	—	—
<i>B. brevis</i>	—	—	—
<i>B. subtilis</i> (Marburg)	—	—	—
<i>B. subtilis</i> (Ford)	—	—	—
<i>B. mesentericus</i>	—	—	—
<i>B. megatherium</i>	—	—	—
O B Williams cultures			
<i>B. cereus</i>	+++	++	++
<i>B. mycoides</i>	++	++	+
<i>B. mycoides</i> variant	+++	++	+
W B Sarles cultures			
<i>B. cereus</i>	+++	++	++
<i>B. mycoides</i>	+++	+	+
<i>B. albolactis</i>	+++	+++	++
<i>B. anthracis</i>	+	+	±
ATCC cultures			
<i>B. albolactis</i>	+++	++	++
<i>B. praussnitzii</i>	++	+	+

## DISCUSSION

*Possible explanation for alterations in egg yolk.* The early work of Osborne and Campbell (1900) on the protein of egg yolk showed that it is largely, if not wholly, a lecithin compound that dissolves in salt solution and behaves like a globulin. Sommer (1935) suggested a hypothesis that depended upon this lecithin-protein

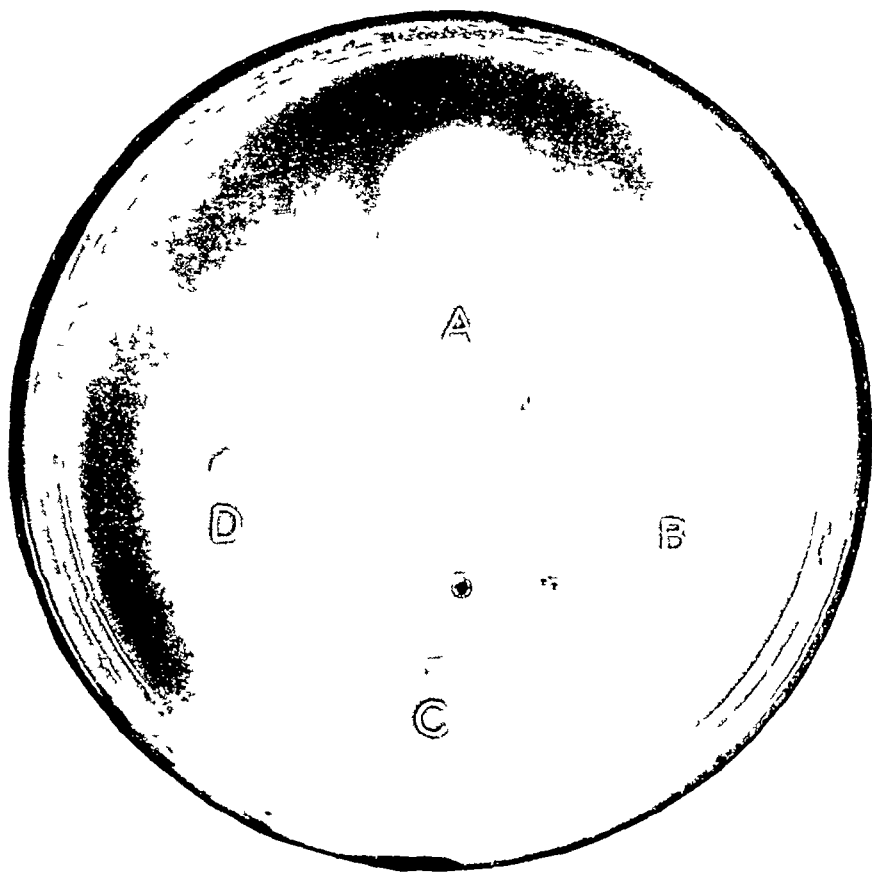


FIG. 1. COLONIES ON LGG YOLK AGAR

A, *B. cereus* (isolate), B, *B. subtilis*, C, *B. mesentericus*, D, *B. megatherium* (Burdon's culture)

complex to explain the action of egg products in increasing the "whip" of ice cream mixes. He stated

Lecithin with its glycerol and two fatty acid radicals in the molecule will dissolve in fat, but because of the other groups in the molecule, will be held at the surface of the fat globules. If the lecithin is in combination with proteins, the lecithin-protein combination will, therefore, cover the surface of the fat globules. The affinity of water for proteins is greater than for fat, such a covered fat globule will then adhere to the serum more tenaciously, the fat globule represents a less serious point of weakness in the lamellae and, therefore, better whipping results.

Probably the yolk of the egg with its balanced system of water, protein, glycerides, phospholipids, etc., is not too far removed from the system studied by Sommer. There is the probability that the lecithinase, acting upon the lecithin of the lecithin-protein fraction of the yolk, breaks down this binder and thus brings about the changes in the colloidal state of the components of the yolk. With the loss of the binder action of the lecithin, the fat and protein change from their dispersed state to that found after the isolate has grown in the egg.

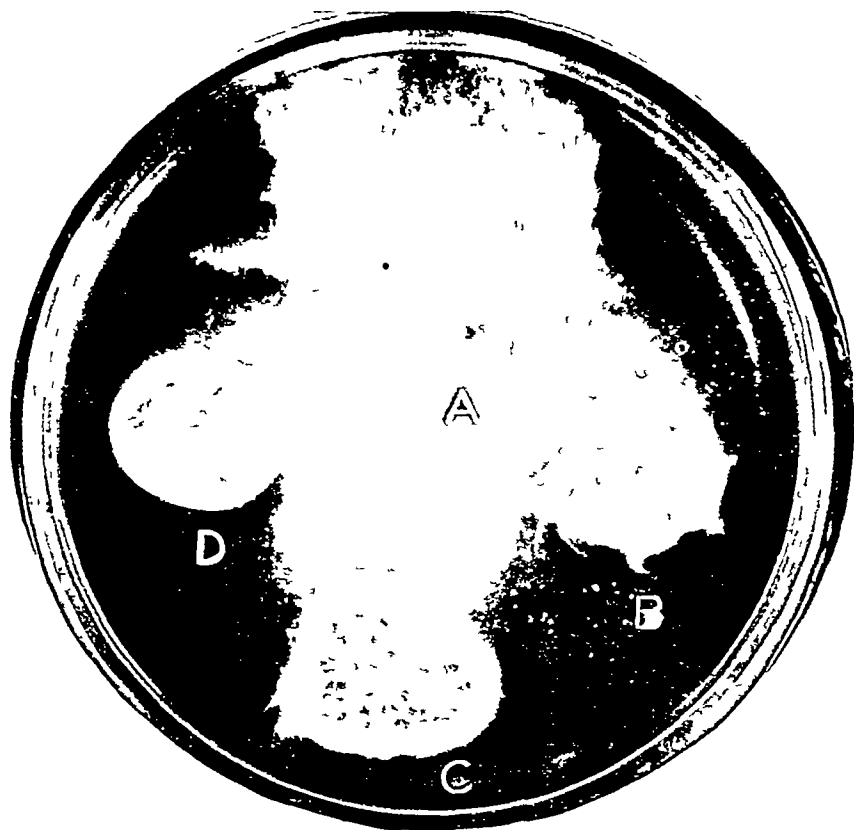


FIG. 2. COLONIES ON EGG YOLK AGAR

A, *B. mycoides* (Sarles's culture), B, *B. praussnitzii* (ATCC culture), C, *B. mycoides* (Williams' culture), D, *B. mycoides* variant (Williams' culture)

In inoculated eggs 10 to 15 days old there appear to be areas of the yolk where they are not as hardened as in other areas. This response of the yolk to the action of lecithinase is in conformity with the findings of Jukes and Kay (1932), who state that the white yolk, in intimate contact with the egg nucleus, is less rich in phospholipids, neutral fat, and protein than the yellow yolk.

Further work is under way to investigate the relation of this lecithinase to those reported by Belfanti, Contardi, and Ercoli (1936).

*Lecithinase activity of some members of the genus Bacillus* A number of workers have noted that some bacteria other than the clostridia can act upon the egg yolk agar and can change the lecitho-vitellin suspension. Hayward (1941) said that in routine tests 3 hemolytic aerobic sporeformers were Nagler-treated and 2 were feebly positive. The same worker (1943) found an aerobic sporeformer that gave to Nagler plates an unneutralized opacity. Weed, Minton, and Carter (1942) found some common sporeformers to give a positive reaction in the lecitho-vitellin

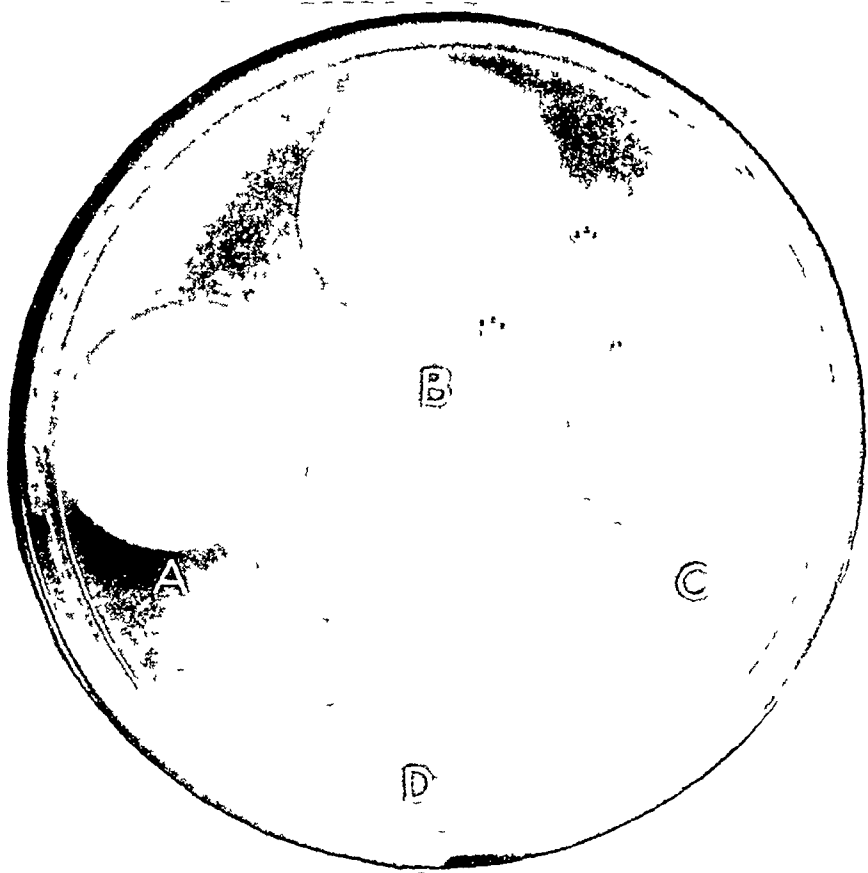


FIG. 3. COLONIES ON EGG YOLK AGAR

A, *B. cereus* (isolate), B, *B. cereus* (Smith's culture), C, *B. albolactis* (ATCC culture), D, *B. albolactis* (Sarles's culture)

suspension. Nagler (1945) stated that a number of species of aerobic sporeformers of the genus *Bacillus* had been tested, but none of them gave the pearly film. McClung and Toabe (1947) obtained positive results with their medium (precipitation but no luster) with strains of *Bacillus* designated as *B. lacticola*, *B. tumefaciens*, *B. ellenbachensis*, *B. megatherium*, *B. cereus*, *B. mycoides*, and several unidentified cultures that appeared as contaminants. They found *B. anthracis* to be among their cultures giving negative results.



It thus appears that the action of aerobic mesophilic sporeforming bacteria on egg yolk components has not gone unnoted. Although the species tested in this work were limited, there is an indication that the possession of the enzyme lecithinase is not widespread among them. Probably wider sampling of authentic cultures might reconcile the reported differences in the possession of lecithinase by some of the members of this genus. Smith, Gordon, and Clark (1946) have

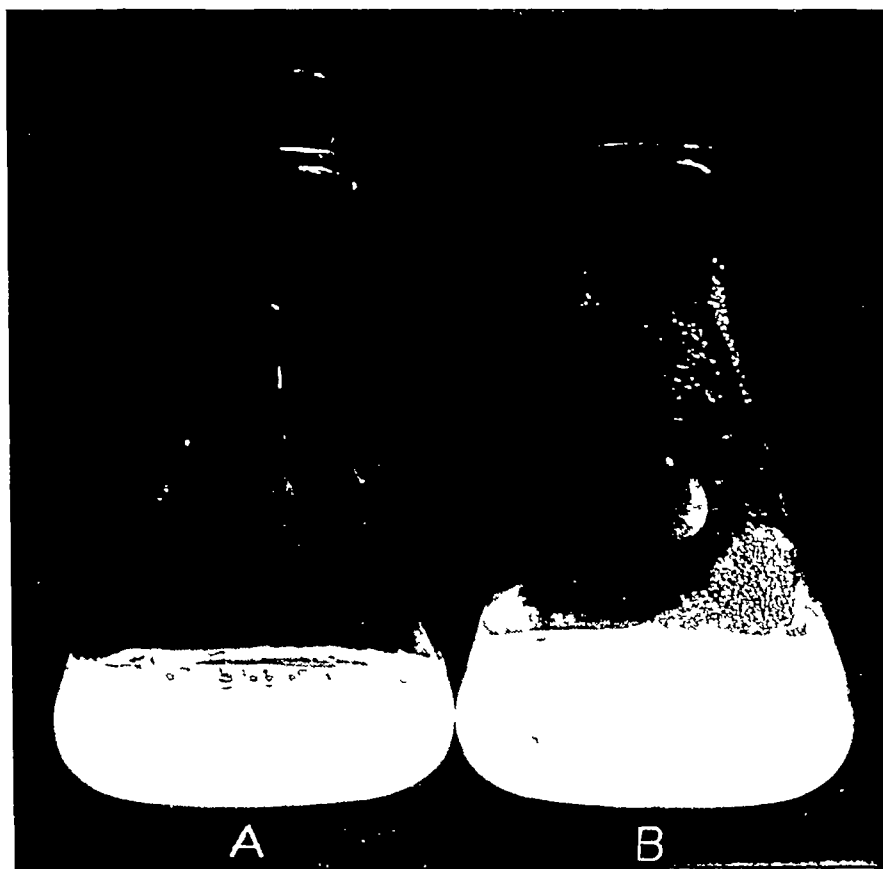


FIG. 4. ACTION OF ENZYME LECITHINASE ON LECITHIN SUSPENSION

A, control flask, lecithin borate suspension with heat inactivated cell free filtrate  
B, lecithin borate suspension after 24 hours' incubation with cell free filtrate

found that some of their cultures labeled *B. lacticola* when received were later identified as *B. cereus*. A similar happening occurred with cultures labeled *B. ellenbachensis*, they, too, were *B. cereus*.

It is of interest to note that the production of lecithinase by two cultures aided in detecting a mislabeling. One culture designated as *Bacillus subtilis* and one designated as *B. megatherium* of the departmental culture collection gave a positive test for lecithinase activity. Later morphological, cultural, and physiologi-

cal tests showed that both organisms were *B. cereus*, confirming the placement originally indicated by their possession of this enzyme. No record of the labeled *B. subtilis* culture was available to show whether it was a *B. subtilis* of the Michigan strain, an organism claimed to be *B. cereus* (Smith, Gordon, and Clark, 1946).

The lecithinase activity of the two cultures of *B. anthracis* was feeble. Where is the zone of precipitation was rather wide about the *B. cereus* colony, with *B. anthracis* the zone extended but little beyond the edge of the colony.

Smith, Gordon, and Clark (1946) have treated in detail certain relationships among the aerobic mesophilic sporeforming bacteria. They state

*B. cereus* possesses quite a wide range of characters and some of its variants and biotypes have been given names as species. For instance, certain strains ferment lactose and have been called *B. albolactis*, *B. lactis*, and *B. lacticola*, others produce a yellowish-green fluorescent pigment and have been called *B. cereus fluorescens* or *B. fluorescens* (not *Pseudomonas fluorescens*), others produce a rhizoid growth on agar, and if they do not ferment lactose they are *B. mycoides*, if they do ferment it they are *B. praussnitzii*. Each one of these characters may be easily lost during studies on dissociation, and the resulting cultures cannot be distinguished from the typical *B. cereus*. The writers consider, therefore, that *B. cereus* is a "parent" or "basic species."

Figure 1 shows the typical reaction on egg yolk agar of a lecithinase producer contrasted with three common nonlecithinase organisms, *B. subtilis*, *Bacillus mesentericus* (*pumilus*), and *B. megatherium*. Figure 2 shows that the enzyme was produced by the rhizoid lactose-fermenting *B. praussnitzii* and the rhizoid non-lactose-fermenting *B. mycoides*. This figure also shows that the nonrhizoid dissociant of *B. mycoides* possessed the enzyme. These last two cultures were from the culture collection of the late I. M. Lewis. Figure 3 indicates that the lactose-fermenting *B. albolactis* and the non-lactose-fermenting *B. cereus* had the same relation to each other in regard to lecithinase as did the rhizoid pair shown in figure 2.

It is felt that the conclusions drawn by Smith, Gordon, and Clark (1946) regarding these organisms and their over-all relation to *B. cereus* can be augmented by the finding that there is a common possession of lecithinase by all of them, a property which does not seem to be possessed outside this group in the genus *Bacillus*.

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#### SUMMARY

Cultures of *Bacillus cereus*, *B. cereus* var. *mycoides*, *B. cereus* var. *anthracis*, and cultures labeled *Bacillus albolactis* and *Bacillus praussnitzii* possessed lecithinase

The possession of this extracellular enzyme by this group of sporeformers is another fact emphasizing their inherent relationship. Other members of the genus that were tested did not produce lecithinase.

It is suggested that the action of lecithinase upon the lecithin fraction of the egg yolk is to destroy its binder action, and the loss of this property causes a marked modification in the colloidal state of the constituents of the egg.

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## VARIATION IN PIGMENT PRODUCTION IN STAPHYLOCOCCUS AUREUS<sup>1</sup>

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The possibility of transforming a specific type of pneumococcus into another type has been recognized since 1928 when Griffith first described this phenomenon. He succeeded in transforming, *in vivo*, an attenuated and nonencapsulated variant into a virulent encapsulated strain of a heterologous specific type. Dawson and Sia (1931) were able to transform pneumococcus types *in vitro*. They used growing R cells in a medium containing anti-R serum and heat-killed encapsulated cells of another type. Later Alloway (1932, 1933) was able to obtain specific conversion of pneumococci by the use of filtered crude extracts of S cells. Berry and Dedrick (1936) converted the virus of rabbit fibroma into that of infectious myxoma in an experiment analogous to that used in pneumococcus transformation. Except for these two specific instances there appears to have been no mention in the literature of attempts at transforming other microorganisms using specific extracts.

During the past few years attempts have been made to isolate and identify the transforming substance. The results of this work indicate that the substance has the characteristics of a desoxyribonucleic acid (Avery, MacLeod, and McCarty, 1944; McCarty, 1946; McCarty and Avery, 1946).

The present study deals with an attempt to induce transformation of a white *Staphylococcus aureus* into a chromogenic type. There are no reports of this transformation having been induced experimentally by a chemically defined substance. However, Pinner and Voldich (1932) do report the reversion of *Staphylococcus albus* into *S. aureus* by growth of the organism in antialbus serum. Nutini and Lynch (1946) were able to induce a strain of *S. aureus* to lose its ability to produce pigment, and during two and one-half years of observation it did not revert to the original orange growth.

### EXPERIMENTAL PROCEDURES

*Staphylococcus aureus* ATC 6538 was chosen as the source material for any possible transforming substance since it is a highly chromogenic organism. *S. aureus* Smith was used as the test organism for conversion; it was nonchromogenic and decidedly mucoid in colony form, and varied in biochemical reactions from the 6538 strain. The Smith strain has been observed over a period of 10 months.

<sup>1</sup> After this work was completed an article appeared in the *Bull. acad. med.*, 131, 39, 1947, by Boivin, Vendrely, and Tulasne, in which the authors stated that they were able to bring about a "directed mutation" of a coliform strain from one antigenic and fermentative type to another by growth in the presence of desoxyribonucleic acid derived from a strain possessing the new characters. Material from the smooth form only was effective.

with no visible evidence of chromogenesis Tests for chromogenic colonies were made on plates of yeast extract agar and on plates of Chapman-Stone medium (1946) Serial passage was made through 10 mice, but again there was no change in the color of colonies

Development of chromogenicity by the colonies of a test organism was accepted as possible evidence of conversion This conclusion was based on the fact that there seems to be no report in the literature of a white *Staphylococcus* spontaneously becoming chromogenic *in vitro*

The test organisms were checked for colony type, ability to liquefy gelatin, to ferment certain sugars, and to produce coagulase Reactions in litmus milk were also observed (table 1)

The requirements for transformation being unknown, certain cultural conditions suggested by Avery, MacLeod, and McCarty (1944) in their work with pneumococcus transformation were accepted These conditions were found necessary for the conversion of pneumococcal types *in vitro* The components included in the transformation test will be described in the following order (1) infusion broth, (2) serum, (3) strain of test organism, and (4) method of preparing the staphylococcal extract

TABLE 1  
Cultural reactions of the test organisms

ORGANISM	PIGMENT	GELATIN LIQUE- FACTION	COAGULASE	FERMENTATION		LITMUS MILK
				Lactose	Mannitol	
<i>S aureus</i> Smith	Grayish white	+	+	-	+	Peptonized
<i>S aureus</i> 6538	Golden	+	+	+	+	Acid

*Infusion broth* Difco beef heart infusion broth, adjusted to an initial pH of 7.2 to 7.4, was used as the basic medium The broth was first adsorbed with charcoal according to the method described by MacLeod and Mirick (1942) before being autoclaved in 250-ml quantities This procedure, originally initiated for the removal of sulfonamide inhibitors, has been reported by Avery, MacLeod, and McCarty (1944) as eliminating to a large extent unpredictable variations in individual lots of broth

*Serum* Beef serum, filtered through a Mandler filter, was used routinely in conjunction with the infusion broth The proportions of serum to broth were 1 to 3, and this mixture was used for maintaining the stock cultures as well as for testing for transforming activity In order to destroy any enzyme that might interfere with a transforming principle the serum was heated to 60 C for 30 minutes

*Test organisms* The organisms used in testing for transformation were grown in two series, one in serum broth and the other in plain adsorbed infusion broth, serial transfers were made daily The Smith strain of *Staphylococcus aureus* showed colonies that were round, raised, opaque, glistening, and so mucoid that colonies touched with an inoculating needle would lift off the agar surface in one

mass or pull out in long mucoid threads. On yeast extract agar these colonies were an off white in color. Sometimes a few dissociant colonies were found on a plate. These, however, were not so opaque nor did they have the characteristic mucoid quality.

*S. aureus* 6538, the organism from which extracts were made, showed an intense orange chromogenesis on Chapman-Stone medium, the color was not quite so intense on yeast extract agar. The colonies were round, raised, and shiny, with an entire margin.

*Method of preparing extract.* A 48-hour chromogenic slant of *S. aureus* 6538 was washed off with broth and this broth suspension used to inoculate Blake bottles. Yeast extract agar was used as the base medium. The Blake bottles were incubated for periods varying from 18 to 24 hours and accepted for further treatment if there was visible evidence of chromogenesis on the slant. Growth on each slant was then washed off in 10 ml of a solution containing 0.1 M sodium chloride and 0.1 M sodium citrate. McCarty and Avery (1946) used this solution to inactivate desoxyribonuclease that had been released with the breakdown of the pneumococcus cell.

Some method had to be devised for rupturing the staphylococcal cells without inactivating any transforming principle that might be present. Chemical agents were tried and seemed to be of slight, if any, value in lysing heavy suspensions of staphylococci. The mechanical method of rapid freezing and thawing was used.

The citrate-saline suspension of organisms was dispensed in sterile test tubes, approximately 6 ml were in each tube. The freezing bath consisted of a beaker, the outside of which was insulated with nonabsorbent cotton. Sufficient amyl alcohol, to more than cover the tube containing the bacterial suspension, was added to the beaker, and pieces of solid CO<sub>2</sub> were placed in the alcohol to hold the temperature well below freezing. The tubes of bacterial suspension were immersed in the freezing bath until frozen solid, removed, and placed in warm water to thaw, then frozen again. This procedure was repeated over a period of 2 hours. At the end of this time gram stains still revealed an abundance of intact gram-positive organisms. Subculturing also indicated that many viable organisms remained. The treated saline-citrate suspension was then deproteinized by a slight modification of the method of Sevag, Lackman, and Smollens (1938). Approximately 5 ml of a chloroform-amyl-alcohol mixture (4 ml chloroform to 1 ml amyl alcohol) were added to each 10 ml of bacterial suspension. The whole was then shaken in a Kahn shaker for 30 minutes, centrifugated for 20 minutes, and the chloroform emulsion removed. The supernatant was again extracted with chloroform-amyl-alcohol, and both fractions were pooled in a separatory funnel. The addition of 2 volumes of alcohol to the extract resulted in flocculation of a fair amount of white material. Citrated saline was then added to the alcohol extract and the whole was well shaken. The funnel was placed in the refrigerator and left there overnight to permit separation of the two phases. The saline extract, which was decanted the following morning, was faintly cloudy, with cloudiness increasing slightly in density as the solution attained room temperature. It is this extract that was used in testing for transforming activity.

*Qualitative chemical tests* The saline extracts gave negative biuret, Millon, and xanthoproteic tests Benedict's test for reducing sugars was also negative The orcinol test (Bial) for pentoses was faintly positive

*Determination of biological activity* In an attempt to obtain optimum proportions, varying quantities of saline extract were added to a series of tubes each containing the same amount of serum broth Each tube in a series was inoculated with a loopful of an 18- to 24-hour broth culture of the test organism The tubes were incubated at 37 C for 24 hours At the end of 24 hours a streak plate, on yeast extract agar, was made from each dilution A loopful of each dilution culture was transferred to another tube containing the same quantity of extract With each determination this procedure was repeated for 5 days in series Control plates of the test organism grown in serum broth were made each day The

TABLE 2  
*Serial transfer of test organism in dilutions of extract*

TUBE NO	EXTRACT	APPROXIMATE PERCENTAGE OF CHROMOGENIC COLONIES PER PLATE				
		1st day	2nd day	3rd day	4th day	5th day
	<i>ml</i>					
1	0 05	—	—	—	—	—
2	0 15	—	—	—	1	3
3	0 25	5	3	1	—	3
4	0 35	—	—	—	30	40
1	0 15	—	—	70	—	—
2	0 20	—	—	—	—	—
3	0 25	—	—	20	50	—
4	0 30	—	—	40	—	—
1	0 15	—	—	—	—	—
2	0 20	—	—	—	—	—
3	0 25	—	—	—	—	—
4	0 30	—	—	—	—	—
1	0 17	—	—	—	—	—
2	0 25	—	—	—	—	—
3	0 32	—	—	50	70	60
4	0 40	—	—	—	—	—

— = no pigmented colonies seen on the plate

plates were grown at 37 C for 48 hours, examined for chromogenic colonies, and then kept at room temperature for 2 weeks before being discarded as negative A stock slant of yeast extract agar was made of any suspected colony, and further studies were made with this stock strain

#### RESULTS

Pigmented colonies were obtained by growing the white *Staphylococcus aureus* Smith in extracts prepared from the highly chromogenic 6538 strain of *S. aureus* Transformation was sporadic and did not occur in all cases In some series no chromogenic colonies developed, in others pigmented colonies were found on plates made from inoculated extract dilutions of the first to the fifth day The results in table 2 are from experiments selected for illustration



Whether the stock culture, used to inoculate extract dilutions, was grown in serum broth or in plain adsorbed broth seemed to have little effect on the results. In many cases colonies did not show pigmentation until there had been a period of incubation at room temperature. A few of the chromogenic colonies were round and smooth, differing from the control colonies only in color. The majority of colonies showed an undulating margin that was more highly pigmented than the body of the colony. Frequently small chromogenic outbursts seemed to develop from a cream parent colony, and transfers made from this margin grew as round pigmented colonies. In no instance was chromogenesis so intense in the new strains as it was in control plates of *S. aureus* 6538, this fact was particularly evident on plates of Chapman-Stone medium. The new ability to produce pig-

TABLE 3  
*Cultural reactions of the isolated strains*

STRAIN	AMOUNT OF PIGMENT	GELATIN LIQUE FACTION	COAGULASE	FERMENTATION			LITMUS MILK
				Glucose	Lactose	Mannitol	
Smith	—	+	+	+	—	+	Peptonized
6538	++++	+	+	+	+	+	Acid
534	++	+	+	+	—	+	Peptonized
755	++	+	+	+	—	+	"
1123	+	—	—	+	—	+	No reaction
1334	+	—	—	+	—	+	" "
1345	+	+	+	+	—	+	Peptonized
1355	++	+	+	+	—	+	"
1353	+	+	+	+	—	+	"
1822	+	+	+	+	—	+	"
1831	++	+	+	+	—	+	"
1833	+	+	+	+	—	+	"
1834	++	+	+	+	—	+	"
1843	++	+	+	+	—	+	"
1853	++	+	+	+	—	+	"
1854	++	+	+	+	—	+	"

ment proved, on ordinary serial transfer, to be unstable, many of the daughter colonies reverting to the original Smith type.

In studies of the newly isolated pigmented strains the colony type resembled those of the Smith organism. There was no variation in sugar reactions. Fermentation of glucose and mannitol was positive, and of lactose negative. Gelatin was liquefied and coagulase was produced by all but two of the least pigmented strains (table 3).

#### CONCLUSIONS

It has been found possible to stimulate or initiate chromogenesis in a nonchromogenic *Staphylococcus* by growing it in extracts of a pigmented strain of the same species. This fact suggests that there might be a transforming principle in the extracts analogous, but not identical, to the transforming principle used to convert *R. pneumococci* into specific types. The extracts used in either case seem

to have similar chemical properties Both give negative protein tests and weakly positive tests for pentose (Bial)

A very small quantity of the transforming substance is capable of initiating a reaction There is no explanation for the fact that some cells of the test organism are refractory to stimulation whereas others are not

#### SUMMARY

An extract has been prepared from *Staphylococcus aureus* 6538, a highly chromogenic organism, which has the potentiality of inducing or stimulating chromogenesis in colonies of a white strain of the same species

The extract prepared gives negative Millon, xanthoproteic, and Benedict tests, and a slightly positive test for pentose (Bial)

The newly isolated chromogenic organisms retain the biochemical properties of the parent culture, differing only in pigment production

The intensity of pigment production is not so great in the new strains as in the strain from which the extract was prepared nor does it seem to be lasting

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# STUDIES OF STAPHYLOCOCCI WITH SPECIAL REFERENCE TO THE COAGULASE-POSITIVE TYPES<sup>1</sup>

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Studies of staphylococci have centered largely around attempts to establish simple laboratory tests that could be used as criteria for the differentiation of pathogenic from nonpathogenic strains. The term "pathogenic," when applied to staphylococci, includes any strain capable, under optimum conditions, of causing an infection or causing a food poisoning.

The best indicator of pathogenic potentiality has been the ability to coagulate oxalated or citrated blood plasma. Other characteristics of some importance are the fermentation of mannitol, orange pigmentation, and the ability to grow in the presence of 7.5 per cent sodium chloride. However, a number of apparently nonpathogenic staphylococci (coagulase-negative) are positive on some of the latter tests.

Evans (1947) has suggested that the anaerobic fermentation of mannitol may be of value for such studies. The introduction of media containing 7.5 per cent sodium chloride by Chapman (1945, 1946a, 1946b) has greatly simplified selective plating for staphylococci. The high salt concentration greatly enhances pigment production by the orange strains, and it inhibits most contaminants with the exception of some sporeforming bacilli.

Nutritional studies of staphylococci have been confined largely to studies on known or supposed pathogens, although in some cases pathogenicity was either not mentioned or not adequately established. Knight (1937) showed that thiamine and nicotinic acid were essential for *Staphylococcus aureus*. Porter and Pelczar (1941) found two mucoid, orange strains from clinical sources that required biotin. Sevag and Green (1944) reported two strains of "toxigenic" staphylococci that required pantothenic acid in the absence of tryptophan, and Vilter and Spies (1940) noted that pyridoxine stimulated growth of a white *Staphylococcus*.

In the present work an attempt has been made to correlate various physiological tests and the nutritional requirements of a collection of staphylococci. The coagulase test has been used as the criterion of pathogenicity. The 19 coagulase-positive strains were from clinical infections, food poisonings, and frozen foods. The 66 coagulase-negative strains were all from frozen foods.

## PHYSIOLOGICAL STUDIES

**Methods** All cultures were carried in beef infusion broth at 35 C. All inoculations were made from approximately 24-hour cultures. In critical tests, such as the coagulase test and mannitol fermentation, the cultures were transferred

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daily for at least 5 days prior to inoculation of the test media. This was particularly important for old stock cultures.

Difco coagulase plasma was used for the coagulase test. A suspension of 0.5 g of the dried plasma was prepared in 15 ml of distilled water. To 0.5 ml of this suspension, in a small tube, were added 2 drops of a 24-hour culture from beef infusion broth. The tube was shaken to mix the culture and plasma and then placed in an incubator at 35 C. Any degree of coagulation within 24 hours was considered positive.

Mannitol fermentation was tested on a medium containing 0.3 per cent beef extract, 0.5 per cent tryptone, 0.5 per cent mannitol, and 0.004 per cent bromcresol purple. For anaerobic fermentation tests, the medium was heated in flowing steam for 15 minutes to drive out dissolved air, and cooled immediately before inoculation. Following inoculation, each tube was sealed with melted "vaspar." Tubes were incubated at 30 C for 10 days. Daily observations were made for changes in the color of the bromcresol purple. Final pH was determined using a Beckman potentiometer. The fermentation of glucose, lactose, sucrose, and glycerol was tested in the same manner.

Pigmentation was determined by inoculating cultures on beef infusion agar slopes and on slopes of Chapman's medium no. 110 (Chapman, 1946a). For cultures that would grow in the presence of 7.5 per cent NaCl, medium no. 110 gave much stronger pigmentation and showed pigment with some cultures that were white on the beef infusion agar slopes.

Stone's extract gelatin agar was inoculated, incubated for 48 hours at 35 C, and tested in the usual manner. Nutrient gelatin contained 0.3 per cent beef extract, 0.5 per cent tryptone, and 4.0 per cent gelatin. It was inoculated with 1 drop of culture, incubated for 10 days at 30 C, and placed in an ice water bath until a control tube was thoroughly solidified.

The phenol red mannitol salt agar was a Difco dehydrated medium containing 7.5 per cent salt, following the suggestion of Chapman (1945). Cultures were streaked on it, and any yellow zone appearing within 48 hours at 35 C was considered positive. The bromcresol purple mannitol salt agar contained 0.3 per cent beef extract, 0.5 per cent tryptone, 7.5 per cent NaCl, 1.0 per cent mannitol, 1.5 per cent agar, and 0.004 per cent bromcresol purple. Cultures were tested by the method used with the phenol red medium.

*Results* The results of the physiological tests are summarized in table 1. The ability to ferment mannitol under anaerobic conditions (Evans, 1947) was possessed by all the coagulase-positive strains, and by only 2 of the 47 coagulase-negative strains that fermented mannitol under the ordinary conditions. These two strains (S-36 and S-37) were isolated from the same plate and were identical in all tests, hence were probably duplicates. They produced an orange pigment, but differed in many other respects from the coagulase-positive cultures. They were negative on the Stone test, failed to grow in the presence of 7.5 per cent salt, and required added thiamine, nicotinic acid, biotin, pyridoxine, and pantothenic acid.

As noted previously (Evans, 1947), the coagulase-positive strains tended to produce only a small amount of acid when grown on the surface of mannitol agar.

containing 7.5 per cent NaCl. With bromocresol purple as the indicator, the coagulase-positive strains produced very little or no yellow zone. This may be

TABLE 1  
*Summary of physiological tests*

	PER CENT POSITIVE		
	Coagulase +	Coagulase—	
		Mannitol +	Mannitol —
Mannitol fermentation			
Aerobic	100	100	0
Anaerobic	100	4	0
Pigmentation			
Orange	89	17	16
Cream	11	9	0
Yellow	0	0	16
Gelatin reactions			
Stone's gelatin	89	6	68
Nutrient gelatin	79	15	74
P R mannitol salt agar	100	96	0
B C P mannitol salt agar	0	85	0
Glucose fermentation			
Aerobic	100	100	95
Anaerobic*	100	90	86
Lactose fermentation	100	64	42
Sucrose fermentation	100	98	74
Glycerol fermentation†			
Aerobic	100	100	—
Anaerobic	0	0	—
Litmus milk reactions			
Acid production	89	19	32
Acid curd	68	2	32
Proteolysis	0	4	16

\* Only 39 strains were tested (11 coagulase-positive, 21 coagulase-negative, mannitol-positive, and 7 coagulase-negative, mannitol-negative)

† Only 18 strains were tested (11 coagulase-positive, and 7 coagulase-negative, mannitol-positive)

due to a more efficient aerobic respiration mechanism, or a greater Pasteur effect. When the 7.5 per cent NaCl was omitted from the medium, more acid was produced and was indicated by bromocresol purple.

## NUTRITIONAL STUDIES

*Methods* Vitamin requirements were determined by omitting the vitamins one at a time from a complete medium composed of the basal medium and the seven B vitamins given in table 2. Enough medium was prepared in one batch to allow for a serial transfer after 24 hours. The medium, which was not used for 24 hours, was steamed for 15 minutes and cooled before inoculation. All tubes were incubated at 30 C. The amount of growth was determined by measuring turbidity with an Evelyn type colorimeter designed for use as a densitometer. The circuits were so arranged that complete light transmission (through a

TABLE 2  
*Medium for nutritional studies*

BASAL MEDIUM	WEIGHT PER 100 ML OF MEDIUM
	mg
Casein hydrolyzate	50
D,L-Tryptophan	1
L-Cystine	1
Glucose	100
K <sub>2</sub> HPO <sub>4</sub>	40
Salts	
MgSO <sub>4</sub> 7H <sub>2</sub> O	2
NaCl	0.10
FeSO <sub>4</sub> 7H <sub>2</sub> O	0.10
MnSO <sub>4</sub> 4H <sub>2</sub> O	0.10
Adenine SO <sub>4</sub>	0.05
Guanine HCl	0.05
Uracil	0.05
Xanthine	0.05
VITAMIN SOLUTIONS	
Thiamine HCl	0.01 mg
Nicotinic acid	0.05 "
Biotin	0.01 µg
Riboflavin	0.01 mg
Pyridoxine HCl	0.01 "
Pantothenic acid (Ca)	0.01 "
Folic acid	0.10 µg

16 0-mm tube of distilled water) gave a reading of zero, and no light transmission gave a reading of 100 on the galvanometer. Permanently installed in the instrument was a red filter transmitting a band of light that centered at about 660 Å. With bacterial cultures, readings from 20 to 90 were approximately linear. Tubes reading below 10 generally showed no turbidity to the naked eye, and tubes of sterile media generally gave readings around 5. The stock solutions were assayed for any contaminating vitamins using streptococci of known vitamin requirements.

*Results* The complete vitamin requirements were determined for 40 strains

TABLE 3  
*Vitamin requirements*

	COAGULASE- POSITIVE	COAGULASE NEGATIVE	
		Mannitol+	Mannitol—
Strains tested	19	18	3
Require thiamine	19	18*	2
“ nicotinic acid	19	16	3
“ biotin	0	18	3†
“ riboflavin	0	0	0
“ pyridoxine	0	2†	0
“ pantothenic acid	0	6	1
“ folic acid	0	0	0

\* Three strains grew to about half-maximum without thiamine

† These were the same two strains that fermented mannitol anaerobically

‡ Two strains grew to about half-maximum without biotin

TABLE 4  
*Biotin requirement*

STRAIN		DENSITOMETER READINGS							
		Complete medium				Minus biotin			
		1st transfer		2nd		1st		2nd	
		24 hr	48	24	48	24	48	24	48
<i>Coagulase +</i>									
	S-1	60	63	64	68	32	42	26	61
	S-6	72	80	63	75	66	77	53	73
	S-14	66	68	68	70	40	67	30	61
	209	67	72	66	70	51	56	44	56
	64	47	52	27	53	26	37	24	50
	99	53	68	60	69	27	55	37	58
	161	37	55	36	56	17	45	23	43
	177	56	62	58	64	27	52	40	52
	185	48	58	56	67	33	57	47	56
	197	33	54	47	59	20	45	30	50
<i>Coagulase — Mannitol +</i>									
	75	56	72	56	69	15	27	6	4
	S-18	55	61	54	64	6	16	9	5
	S-19	36	61	38	64	10	29	9	19
	S 37	31	63	10	65	3	10	5	7
	13-12	30	61	55	61	8	15	10	14
	13-20	59	75	54	71	8	9	6	4
	15-1	52	55	50	58	10	15	7	4
<i>Coagulase — Mannitol —</i>									
	8-10	6	48	9	50	6	6	6	6
	20-1	32	71	54	72	8	45	6	36
	22-7	26	76	43	78	7	38	5	23

The complete medium contained the usual base plus the 7 vitamins The other medium was identical except for biotin

The results are summarized in table 3. It will be noted that the ability to grow well in the absence of added biotin was limited to the coagulase-positive strains.

An additional experiment covering the biotin requirement of the 19 coagulase-positive strains and 29 of the coagulase-negative strains gave essentially the same results. Turbidity readings for 20 of these cultures are given in table 4. The cultures for which data are not given gave very similar readings. Biotin exerted a mild stimulatory effect on some of the coagulase-positive strains, particularly in the first 24 hours of growth. Also, some of the coagulase-negative strains were able to grow slightly without added biotin, but a wide difference in growth response between the two groups is still obvious. This was even more striking when the strains unable to ferment mannitol were eliminated. Since Porter and Pelczar (1941) did not give any physiological data on their two strains

TABLE 5  
*Effect of omissions from the basal medium*

	COMPLETE	MINUS TRYPTOPHAN	MINUS CYSTINE	MINUS PURINES AND PYRIMIDINES
<i>Coagulase +</i>				
209	69	68	34	61
64	58	53	28	52
99	71	68	31	68
S-6	79	75	50	74
S-14	69	68	36	59
177	75	61	35	65
197	61	61	32	43
<i>Coagulase -</i>				
75	69	67	65	67
S-18	58	59	52	53
13-20	73	36	56	68

Densitometer readings of 10 mannitol-fermenting strains (second transfer, 48 hours) showing the effect of omitting components from a complete medium (basal plus thiamine, nicotinic acid, and biotin)

which required biotin, it is possible that they may have been coagulase-negative.

The effect of omitting some components of the basal medium is shown in table 5. There was no appreciable effect when tryptophan was omitted except on one coagulase-negative strain, which achieved only about half-maximum growth. Cystine appeared to be definitely stimulatory to the coagulase-positive strains, under the conditions used. The omission of adenine, guanine, uracil, and xanthine had little or no effect on the growth of the 10 strains tested. All of this would be expected in view of the work of Surgalla and Hite (1946) and Surgalla (1947), who grew enterotoxic strains in a medium containing only arginine, cystine, glucose, salts, nicotinic acid, and thiamine.

#### DISCUSSION

Although the strains included in this study are admittedly few in number, the results indicate that the staphylococci that give a positive coagulase reaction



comprise a rather homogenous group. They ferment mannitol, tend to produce pigment, generally give a positive Stone reaction, ferment glucose, lactose, sucrose, and glycerol, and require added thiamine and nicotinic acid. All of the foregoing characteristics have been emphasized by previous investigators, but there are many coagulase-negative strains having several or all of these characteristics. They may be separated, however, by the ability of the coagulase-positive strains to ferment mannitol under anaerobic conditions and to grow in a synthetic medium devoid of biotin. These results appear to be contradictory to those of Colwell (1939), who reported 23 orange-pigmented strains from clinical sources to be unable to ferment mannitol anaerobically. However, the coagulase test was not run on those cultures and their pathogenicity was not clearly established. Also, it should be pointed out that active cultures are essential to this test, and stock cultures should be serially transferred for several days before testing.

The pantothenic acid requirement of 7 coagulase-negative strains was shown in the presence of tryptophan. No attempt was made in the present work to establish a pantothenic acid requirement in the absence of tryptophan, as was demonstrated by Sevag and Green (1944). However, in the experiment reported in table 5, when tryptophan was omitted from a medium that contained no pantothenic acid, only one culture (coagulase-negative) showed any significant decrease in growth. Other tests had shown this batch of casein hydrolyzate to be free of tryptophan.

The requirement for pyridoxine shown by strains S-36 and S-37 was unique among the cultures studied. Vilter and Spies (1940) reported that pyridoxine was stimulatory for one strain of *Staphylococcus albus*, but the present report seems to be the first record of pyridoxine being essential for a *Staphylococcus*.

The phenol red mannitol salt agar (Chapman, 1945) proved to be a satisfactory selective plating medium, although it was observed that some sporeforming bacilli were able to grow on this medium when it was used for checking frozen foods. Also, a number of coagulase-negative staphylococci grew on the medium and produced acid. No experiments have been directed toward incubating these plates under anaerobic conditions, although this technique might prove to be superior for both differential and selective purposes.

#### SUMMARY

The 19 coagulase-positive staphylococci studied comprised a rather homogenous group. They could be separated from the 66 coagulase-negative strains studied by their ability to ferment mannitol under anaerobic conditions, and their ability to grow in a synthetic medium devoid of biotin.

Among the coagulase-negative staphylococci, 6 strains were found to require pantothenic acid and 2 required pyridoxine. These requirements appear to be unique among the staphylococci thus far reported.

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# STAPHYLOCOCCAL PENICILLINASE CHARACTERISTICS OF THE ENZYME AND ITS DISTRIBUTION

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The penicillin inhibitor or penicillinase produced by certain strains of *Staphylococcus* has been the subject of experimentation in a number of laboratories. There is general agreement that the enzyme is present in many but not all strains of *Staphylococcus*, and it has also been held to be responsible for the "natural" resistance of some strains to the action of penicillin. In some studies characteristics have been attributed to the enzyme that are very difficult to explain in terms of the body of knowledge of enzyme chemistry.

The demonstration of penicillinase did not long follow the beginning of intensive work on the antibiotic itself. Abraham and Chain (1940) were probably the first to call attention to its presence, and Harper in 1943 described a practicable method for its demonstration within the cells of bacteria. His method, of acetone and ether treatment of whole bacterial cells, has been used by all subsequent investigators. In their first studies, Bondi and Dietz (1944a,b) failed to find the enzyme in *Staphylococcus*. Later (1945), examining more strains, they demonstrated its presence in 14 per cent of them. They record the enzyme as present or absent, and find it invariably present in strains requiring more than 1 unit per ml of penicillin for inhibition. Kirby (1945) in a brief note stated that he had found penicillinase, using Harper's method, in 7 resistant strains, but not in 7 sensitive strains of *Staphylococcus*. Later 5 of the resistant, penicillinase-producing strains were studied in detail. The enzyme was assayed by adding the penicillinase, penicillin, and a culture of viable penicillin-sensitive organisms to nutrient broth. The growth of the indicating bacteria was followed turbidimetrically. On the basis of such assays, it was stated that the rate of action of the enzyme appeared to be independent of temperature between 2 and 37 C.

Spink and Ferris (1945) reported after a preliminary study of 8 strains that organisms that acquired penicillin resistance *in vivo* produced penicillinase, whereas those whose resistance was induced in the laboratory did not. Later (1947), in an exhaustive study, these results were confirmed. The method of assay used by these workers consisted of adding 0.001, 0.01, or 0.1 mg of penicillinase to tubes of broth containing various quantities of penicillin. An inoculum of a penicillin-sensitive staphylococcus was then added, and the tubes were incubated for 48 hours. The destruction of penicillin was indicated by the development of turbidity after incubation. By this method they were able to demonstrate that the destruction of penicillin by penicillinase did proceed with time, but they were able to show only a rough correlation between the inactivation of penicillin and the concentration of penicillinase, a result that was at-

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tributed to the impurity of the preparation. A correlation between the potency of the penicillinase formed and the resistance to penicillin exhibited by a given strain was also demonstrated in this way. Although the correlation was rather rough, the authors attribute this to the use of crude material, and conclude that the magnitude of the resistance to penicillin manifested by permanently resistant strains is quantitatively related to the potency of the penicillinase produced by the strain.

Because of the importance of the subject from both practical and theoretical aspects, the problem was reinvestigated using exact methods. It was planned to develop a precise method of assay of the material, and to apply this to the study of a number of strains. Both of these objects have been accomplished.

#### METHODS AND MATERIALS

*Strains of Staphylococcus* The staphylococcal strains used in these experiments were isolated from clinical material in the routine diagnostic laboratory of the Lakeside Hospital, and have been the subject of previous studies. Following isolation they have had a few transfers on artificial media and have been preserved by desiccation from the frozen state.

*Tests of penicillin resistance* For determining resistance to penicillin, standard practice has been followed in preparing serial twofold dilutions of a penicillin solution in tryptose broth, inoculating each tube with the organism being tested, and incubating for 18 hours. At the end of that time each tube is inspected for macroscopic evidence of bacterial growth, and the lowest concentration of penicillin that causes complete inhibition of visible bacterial growth is recorded as the "inhibiting concentration." These tests have been done in duplicate, using "small" and "large" inocula. The small inoculum consists of 0.5 ml of a  $10^{-8}$  dilution of an 18-hour culture in tryptose broth (about  $10^2$  viable cells) and the large inoculum of 0.5 ml of a  $10^{-2}$  dilution. The final volume of the test was 1.0 ml. Resistance to penicillin as measured by using a "small" inoculum is designated R<sub>6</sub>, with the "large" inoculum R<sub>2</sub>.

*Index of penicillin resistance* In an earlier study (Parker, 1946) it was desired to have a simple method of designating the ratio between the R<sub>6</sub> and R<sub>2</sub> results. The "index of penicillin resistance" is the difference expressed as the number of twofold dilutions between the R<sub>6</sub> and R<sub>2</sub> results.

*Culture medium* Tryptose broth and tryptose agar have been used as culture media. Incubation was at 37°C.

*Penicillin* In some earlier experiments amorphous penicillin of relatively high purity was used. For most of them, crystalline penicillin G (CSC) has been employed.

*Preparation of penicillinase* The method of Harper has been followed. It was learned, however, that more consistently potent preparations could be secured if the manipulations were carried out at a low temperature, and the following slightly modified technique has been adopted as standard.

Bacteria from 2 liters of a 22- to 24-hour culture of staphylococci were thrown down by centrifugation and suspended in about 6 ml of Locke's solution. This

bacterial suspension was added with constant shaking to about 50 to 60 ml of acetone, previously cooled to  $-20^{\circ}\text{C}$ . The mixture (in a centrifuge tube of 70-ml capacity) was placed in a refrigerator at  $-20^{\circ}\text{C}$ . At the end of approximately 1 hour the clear supernatant liquid was removed by suction, replaced by fresh cold acetone, and the sedimentation at  $-20^{\circ}\text{C}$  repeated. After 2 such resuspensions in acetone, ether was substituted, and the bacteria were allowed to sediment once more at  $-20^{\circ}\text{C}$ . The ether was drawn off, replaced with a fresh quantity, and the ether suspension of bacteria was allowed to come to room temperature. As much of the ether as was possible was then drawn off, the remainder was allowed to evaporate, and the resultant whitish powder was transferred to a weighing bottle. This was placed over "dri rite" in a vacuum desiccator for 24 to 48 hours before being used.

The resultant powder is referred to in these experiments as "penicillinase," following the common practice. The powder is, of course, composed simply of defatted but otherwise intact bacterial cells, as can be shown with the microscope. In a strict sense penicillinase has not been prepared, but organisms have been so treated as to reveal its presence in the still intact cell.

*Penicillin assay* The cup plate method was used for all penicillin assays. *Bacillus subtilis* (kindly provided by Dr. Foster of Merck and Company) was used as the test organism, 4 cups were used for each determination and incubation was for 18 hours at  $28^{\circ}\text{C}$ . A control curve was determined for each run, the results of this being plotted to the best straight line on semilogarithmic graph paper.

#### EXPERIMENTAL RESULTS

The first experiments were designed to determine the relation of the penicillin-inactivating enzyme to the bacterial cell and the quantitative effects of controlled variation of temperature, time, concentration of enzyme, and concentration of penicillin at pH 7.0. Further experiments were designed to test the reproducibility of results with a given preparation, and with several preparations from a given strain. It was considered necessary to obtain these data before embarking on a program of assaying the penicillinase content of a number of different strains of *Staphylococcus*.

*Experiment 1* An initial experiment was done to determine whether appreciable amounts of penicillinase could be removed from the cells by extraction in McIlvaine's buffer.

A suspension of penicillinase was prepared containing 1 mg per ml. One portion of this was centrifuged for 1 hour at 3,000 rpm (1,600 g) and the supernatant carefully pipetted off. Another portion was filtered through a sintered glass filter, and the filtrate collected. Appropriate quantities of the original suspension, of the supernatant after centrifugation, and of the filtrate were added to 1-ml volumes of penicillin solution. The volumes of all were adjusted to 20 ml, and each was titrated by the cup plate method. The results are presented in table 1.

It is seen that much activity was removed by centrifugation, and all by filtra-

tion The results were not altered by preparing the suspension and allowing extraction to proceed for 48 hours at 5 C, or by substituting a Seitz for the glass filter

*Experiment 2* Since it had been shown that the enzyme could be removed from a system by passage through a bacterial filter, it was planned to determine the rate of destruction of penicillin at 37 C by a constant quantity of enzyme

A suspension of penicillinase containing 0.4 mg per ml was brought to 37 C in a water bath To this was added an equal volume of penicillin solution containing 20 units per ml (previously brought to 37 C) to make the final concentration of penicillin 10 units per ml and of penicillinase 0.2 mg per ml After 30 and 60

TABLE 1  
*Attempted aqueous extraction of penicillinase*

TREATMENT OF SUSPENSION	QUANTITY MIXED WITH PENICILLIN	PENICILLIN IN MIXTURE	
		Originally	Assay after mixing
None	0.2	5.0	0.53
Centrifuged (supernatant added)	0.5	5.0	1.0
Filtered (filtrate added)	1.0	5.0	5.3

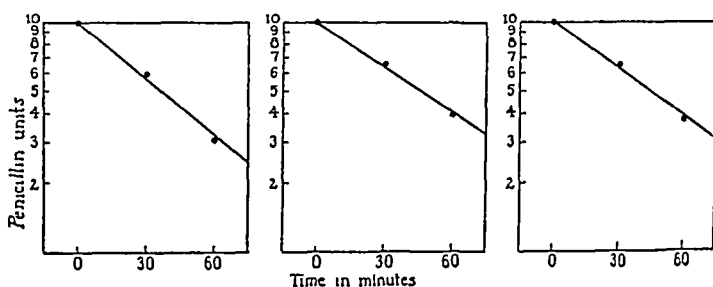


FIG 1 EXPERIMENTS ILLUSTRATING THE CONSTANCY OF THE RATE OF DESTRUCTION OF PENICILLIN AT 37 C

minutes portions were removed and quickly passed through Seitz filters, and the filtrate was assayed for penicillin

In figure 1 the results of three such experiments are presented It was concluded from these and similar experiments that the destruction of penicillin by penicillinase proceeded regularly with time

*Experiment 3* This experiment was designed to determine the effect of temperature on the rate of destruction of penicillin

A suspension was prepared containing 0.2 mg per ml of penicillinase After equilibration at an appropriate temperature in the water bath, an equal volume of penicillin solution, 10 units per ml, was added Samples were removed at intervals, passed through Seitz filters, and the filtrates tested for penicillin

The results are portrayed in figure 2 It is evident that, within reasonable limits, the rate of the reaction is a function of the temperature

In further experiments it was shown that at a given temperature and initial concentration of penicillin the rate of destruction was directly proportional to the concentration of penicillinase, and the results of a typical experiment are shown in table 2

On the basis of the experimental results detailed above, it seemed reasonable to conclude that the destruction of penicillin by the acetone-ether-treated cells of *Staphylococcus* was regular and followed the general characteristics of an enzymic reaction. It seemed reasonable also to assume that, by application of the techniques described above, it should be possible to assay the penicillinase content of a mass of bacterial cells with a reasonable degree of accuracy. For

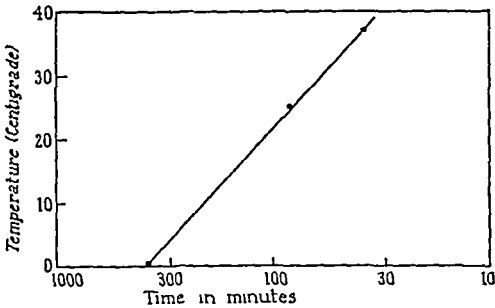


FIG. 2. EXPERIMENTS ILLUSTRATING EFFECT OF TEMPERATURE ON RATE OF REACTION

Quantity of enzyme and original concentration of enzyme constant. "Time" is the time required for 50 per cent destruction of the penicillin.

TABLE 2  
*Destruction of penicillin by penicillinase*  
(Effect of concentration of penicillinase)

MG PENICILLINASE PER ML	UNITS PENICILLIN PER ML	TIME FOR 50% DESTRUCTION
		min
0.1	10	24
1.0	10	2.3

the purposes of these experiments, one unit of penicillinase is defined as the quantity required to reduce the concentration of a solution of penicillin from 10 units per ml to 5 units in 30 minutes at 37 C and at pH 7. Before proceeding to the second part of the study, namely, the survey of a number of strains of *Staphylococcus* for penicillinase activity, it was decided to test the reproducibility of the assay when applied to several preparations of a single strain of *Staphylococcus*.

Five preparations were made from a single strain of *Staphylococcus* (strain 2). The cells in 2 liters of a 22- to 24-hour culture in tryptose broth were centrifuged down and resuspended in Locke's solution. The concentrated bacterial suspension was divided into 2 roughly equal portions, and each was then processed and

assayed independently. Five pairs of preparations were thus secured, from cultures made on 5 different days. The results of the assays of these preparations are given in table 3.

It is seen that excellent agreement was obtained, both between the pairs of preparations from the same culture and between preparations made from different cultures. On the basis of these results, it appeared reasonable to conclude that, by controlling the conditions and duration of action of penicillinase on penicillin, the quantity of the former present in a given preparation could be determined with considerable precision. Further it appeared that the quantity of enzyme present in the cells of a culture was reasonably constant.

*The Distribution of Penicillinase in Strains of Staphylococcus*

On the basis of the results described above, indicating that the quantity of penicillinase in a preparation could be measured with reasonable accuracy and

TABLE 3  
*Replicate assays of Staphylococcus aureus, strain 2, for penicillinase*

PREPARATION	UNITS PER MG
3/18/47—A	14.3
B	12.5
3/19/47—A	16.7
B	14.3
3/20/47—A	16.7
B	17.9
3/21/47—A	15.2
B	15.2
3/25/47—A	16.7
B	16.7

that several subcultures of a given strain did not seem to affect the quantity of penicillinase contained, it was considered feasible to proceed to the assay of the penicillinase content of a number of strains of *Staphylococcus* cultivated under comparable conditions.

Selection of the strains to be tested was guided to some extent by the results of previous investigations of organisms in the collection. It was desired to gain information, if possible, on the mechanism of the "postpenicillin dormant period" exhibited by this organism after short exposure to penicillin, and to determine whether the apparently greater resistance of *Staphylococcus* when heavy inocula were used in testing was due to the presence of penicillinase. A total of 10 strains were examined, and the pertinent data are collected in table 4. In order to facilitate their analysis certain of them are also presented in graphic form.

It was shown in previous studies (Parker and Marsh, 1946) that a culture of *Staphylococcus*, exposed for a very short time while in the logarithmic period of



TABLE 4  
Survey of 40 strains of *Staphylococcus* for penicillinase

NO	R6*	R2†	1‡	UNITS/MG	LOG UNITS/G
35	0 12	0 12	0	—§	—
140	0 12	0 12	0	—	—
69	0 12	0 25	1	—	—
150	0 12	0 25	1	—	—
104	0 12	1 0	3	50 0	4 70
18	0 12	31 0	8	20 0	4 30
70	0 12	500 0	12	125 0	5 10
91	0 12	500 0	12	100 0	5 00
48	0 25	2 0	3	4 17	3 60
54	0 016	0 25	4	—	—
136	0 06	8 0	7	33 3	4 52
146	0 016	0 06	2	—	—
3	0 016	0 25	4	—	—
76	0 016	0 016	0	—	—
9	0 03	0 03	0	—	—
1	0 06	0 06	0	0 22	2 34
25	0 06	0 06	0	—	—
126	0 25	125 0	9	2 2	3 34
7	0 016	0 06	2	—	—
16	0 25	500 0	12	5 0	3 70
8	0 25	500 0	12	20 9	4 32
31	0 5	250 0	9	12 5	4 10
114	0 5	31 0	6	16 7	4 21
68	0 5	125 0	8	2 27	3 36
81	4 0	8 0	1	0 69	2 84
85	4 0	8 0	1	0 19	2 28
2	62 0	250 0	2	16 7	4 21
37	62 0	125 0	1	11 1	4 04
53	0 008	0 06	2	—	—
65	0 008	0 06	2	—	—
83	0 03	0 25	3	—	—
144	0 06	1 0	4	—	—
117	0 06	1 0	4	20 0	4 30
143	0 06	1 0	4	—	—
166	0 06	0 25	2	—	—
141	0 06	0 25	2	—	—
34	0 03	0 125	2	—	—
138	0 06	0 25	2	—	—
132	0 06	0 25	2	—	—
58	0 03	0 125	2	—	—

\* R6, the inhibitory concentration of penicillin on overnight test, when a  $10^{-8}$  dilution of culture was used to inoculate the test series

† R2, the same as R6, but  $10^{-2}$  dilution used as inoculum See text

‡ "Resistance index" The difference, expressed as number of twofold dilutions, between R6 and R2

§ Quantity of penicillinase in arbitrary units in 1 mg of dried powder (—) indicates that if any penicillinase was present, it was in a concentration of less than 0.004 units per mg

growth to an appropriate concentration of penicillin, the penicillin then being removed, entered a second stationary period, followed later by resumption of logarithmic growth. The idea was entertained that this "postpenicillin stationary period" represented the time required to destroy the penicillin that had

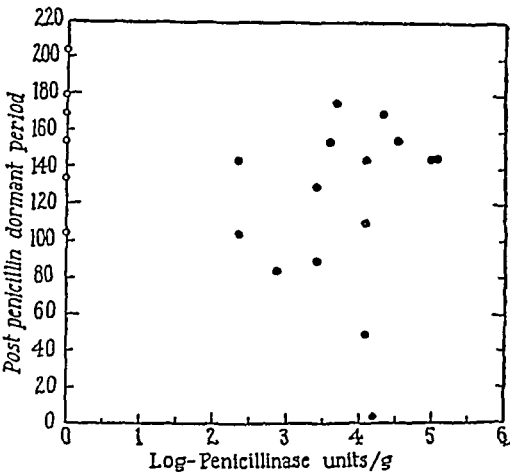


FIG 3 RELATION BETWEEN THE DURATION OF THE POSTPENICILLIN DORMANT PERIOD IN MINUTES AND THE QUANTITY OF PENICILLINASE IN A CULTURE

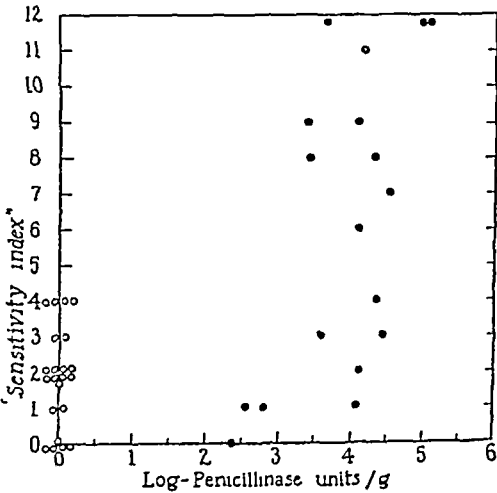
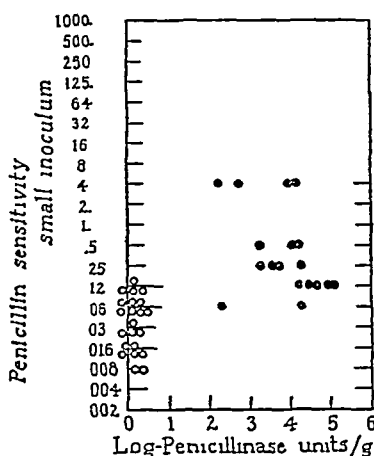
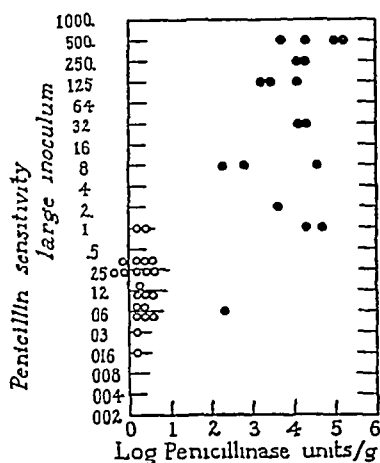


FIG 4 RELATION BETWEEN "SENSITIVITY INDEX" OR "RESISTANCE INDEX" AND PENICILLINASE CONTENT

entered the bacterial cell. It is seen from figure 3, however, that the post-penicillin stationary period has no relation to the penicillinase content of the organism.

Duplicate assays of the penicillin resistance of a number of strains of *Staphylococcus* indicated that, though in most cases the result was little influenced by the

size of the test inoculum, in an appreciable number this was of great importance (Parker, 1946) Luria (1946), on the basis of similar data and rough tests for the presence of penicillinase, concluded that the discrepancy was due to the production of penicillinase by certain strains. In figure 4 the data required to



FIGURES 5 (UPPER) AND 6 (LOWER) RELATION BETWEEN PENICILLIN SENSITIVITY OF INDIVIDUAL STRAINS, AND THEIR PENICILLINASE CONTENT

Open circles indicate strains with no detectable penicillinase

test this hypothesis are collected. It is seen that in those strains with the greatest "index of resistance," i.e., showing a greatly increased "resistance" when a large test inoculum was used, the penicillinase content is high. But, on the other hand, a number of strains that produced penicillinase in quantity did not

show the phenomenon, having an index of 0 or 1. Further, several strains showing 8- to 16-fold differences between the two tests of resistance ("indices" of 3 and 4) produced no measurable amounts of enzyme. The use of the "index" alone as a means of selecting penicillinase producers thus proved to be of very limited value.

Spink has suggested that it is the penicillinase content of a cell that largely determines the resistance of those strains possessing "natural" or "permanent" resistance. The data of figure 5 appear to agree fairly well with this hypothesis. The inocula in these tests of resistance were of the order of 9 million cells per ml of test medium. When the inoculum was reduced to 900 cells, the correlation between resistance and penicillinase content changes very definitely (figure 6). There is still a difference between the resistance, on the average, of those strains possessing enzyme and those possessing none, but among those strains having a measurable amount of penicillinase, there is no significant correlation between degree of resistance and quantity of penicillinase.

#### DISCUSSION

In agreement with the observation of others, it has been shown that many strains of *Staphylococcus* produce a potent penicillinase, and a method is described, consisting essentially of acetone and ether extraction of bacterial cells at low temperature, for securing preparations of uniform activity from a given bacterial strain. It has been shown that the rate of destruction of penicillin by such preparations is regularly related to temperature, time, and concentration of enzyme, as would be expected. It has not proved possible, in limited experiments, to separate the enzyme from the bacterial cell by means of aqueous extraction at pH 7.0.

Inasmuch as the enzyme can easily be moved from a reacting mixture by passage through a Seitz filter, and the time of its action therefore controlled, it lends itself readily to exact assay. In the experiments reported here, one unit of staphylococcal penicillinase has been defined as the quantity required to reduce the concentration of 1 ml of a solution of penicillin from 10 units to 5 units in 30 minutes at 37 C and at pH 7.0. It is perhaps worth emphasizing that the time of action of any enzyme on its substrate must be subject to control, before exact assays can be done. Using the method of assay described above, the penicillinase content of 40 strains of *Staphylococcus* has been determined, and the results are recorded. The strains appear to fall into two groups: (1) strains having no penicillinase, or at least less than 4 units per g, and (2) strains having 190 or more units per g. Among the penicillinase-producing group of strains, of which there were 18, the variation was wide, the range being from 190 units per g to 125,000 units per g. It is altogether probable that examination of more strains would extend this range. Enough have been examined, however, to indicate that it may not be sufficient for all purposes to divide staphylococci simply into penicillinase producers and non-penicillinase-producers.

It has been suggested by a number of workers that penicillinase production is related to penicillin resistance in *Staphylococcus*, and this appears on first ex-

amination to accord with our data. The correlation between resistance and penicillinase content is quite definite when the test inoculum consists of some  $10^6$  cells per ml, and there is good correlation not only between degree of resistance and the presence or absence of penicillinase, but also between the degree of resistance and the quantity of penicillinase contained in the cell. However, when the inoculum is reduced to the order of a few hundred cells, the latter correlation disappears. In view of this, and of the very large reduction in the average resistance to penicillin (from about 31 units per ml to about 0.25 units) with reduction of the inoculum, it seems reasonable to suppose that the "resistance" to penicillin exhibited by penicillin-resistant cells is an artifact introduced by the method of testing; it expresses simply the ability of the resting cell to dispose of the penicillin in its environment. With the disappearance of the penicillin, bacteriostasis is terminated and multiplication begins.

It is to be noted that in the case of those strains producing "no penicillinase" there is a modest although probably significant drop in apparent resistance as measured *in vitro* from an average of about 0.12 units per ml to about 0.03 units per ml when the smaller inoculum is used. The net effect, however, is to reduce the discrepancy between the "average resistance" of the two groups of organisms from a ratio of about 250 to 1 to about 8 to 1, and it seems altogether likely that if the penicillin could be maintained in the test tube, in constant concentration, the difference would disappear entirely. The "resistance" that is conferred on an organism by the possession of penicillinase thus proves to be largely an artifact, important only when the number of cells is large and the quantity of penicillin limited. The discrepancy between the penicillin sensitivity, as measured using large and small inocula, that exists in those strains without demonstrable penicillinase (and probably, but to an undeterminable extent, among those with the enzyme) is probably an expression of the variation in sensitivity to penicillin that normally exists between the cells of a culture. Evidence exists that not all cells of a culture, even when this is derived from a single cell, possess the same sensitivity to various antibiotics. With the use of a large inoculum, containing many cells, the chance of including an unusually resistant one in the inoculum is increased in proportion to the size of the inoculum.

#### SUMMARY

A method for the preparation of staphylococcal penicillinase is described that consists essentially of acetone-ether treatment of staphylococcal cells at low temperature.

By controlling the duration of its action on penicillin as well as other conditions, accurate assay of staphylococcal penicillinase is possible. A method is described that yields reproducible results.

The results of exact assay of 40 strains of *Staphylococcus* for penicillinase are presented. When a small inoculum is used for testing the sensitivity of a strain to penicillin, the sensitivity appears not to be correlated significantly with the content of penicillinase of the same strain.

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# FACTORS AFFECTING THE PRODUCTION OF AMYLASE BY *ASPERGILLUS NIGER*, STRAIN NRRL337, WHEN GROWN IN SUBMERGED CULTURE

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The use of fungal amylase on a controlled scientific basis began when Calmette in 1894 isolated an active amylolytic mold from the crude rice cake of commerce known as Chinese yeast. Although Chinese yeast contained many types of molds and other microorganisms, several amylolytic molds were found to be the active principle of these cakes and from them Calmette finally selected a member of the *Rhizopus* group. Spores of this organism may be developed on sterilized rice and propagated in tanks of successively progressing volume in a liquid cereal medium.

The growing medium is prepared by treatment of these mashers with from 0.3 to 1.0 per cent HCl or H<sub>2</sub>SO<sub>4</sub> followed by autoclaving and neutralization to a pH of 5.0. This provides a medium in which solubilization of starch and reduction of viscosity caused by the acid cooking encourage rapid propagation of mold mycelium when the mashers are agitated with sterile air. Under these conditions the *Rhizopus* converts the starch to sugar and, more slowly, to ethyl alcohol. In practice, the mold is grown with a yeast that gives a more rapid and therefore more practical alcoholic fermentation. This is essentially the amylo process, descriptions of which have been given by Grove (1914), Foth (1929), Boulard (1916), Delemar (1919), and Owen (1933). A modified amylo process has been described by Erb and Hildebrandt (1946) in which rigorous sterility is maintained in developing mold in an amount equal to approximately 10 per cent by volume of the final fermentation. The final fermentation is prepared by conventional methods except that the malt is sharply reduced. No extraordinary sterility precautions are taken in the large fermenters. Under these conditions the additive effect of the 10 per cent pure culture of mold and the small amount of malt (1 to 2 per cent) gave significant increases in the yield of alcohol over those obtained when the conventional amount of malt was used alone.

The amylo process has the advantage that "submerged growth" is employed. This is easily accomplished in a distillery by agitation of the mash in closed vessels by means of air sparged into the tanks at the bottom. Its disadvantage lies in the absolute necessity of pure culture equipment and personnel especially trained in its operation.

Fungal amylases have been developed by Takamine (1914), Underkofler *et al* (1939, 1941, 1942, 1943), and Christensen (1944), who utilized surface

growth of amylolytic mold upon a solid substrate, usually acidified and sterilized wheat bran. These preparations require considerable equipment in the form of trays, driers, etc. The organism usually employed for the production of mold bran is *Aspergillus oryzae*. The product has been found to be equal if not superior to malt for the production of ethyl alcohol from grain. A distillery must, of course, either purchase this material, which adds to the cost of its product, or erect a separate plant for its production.

In the present work a culture of *Aspergillus niger* (NRRL237) obtained from the Northern Regional Research Laboratory was used. This culture was found by Le Mense, Corman, Van Lanen, and Langlykke (1947) to be the organism of choice when 250 amylolytic cultures were screened. It differs from the *Rhizopus* cultures used in the amylo process in that it produces larger amounts of alpha amylase and of maltase than do the conventional amylo molds. Larger amounts of maltase are also produced by *A. niger* 337 than by molds usually employed in the manufacture of mold bran.

In this paper the effect of various factors on the production of amylase by *A. niger* 337 is described. The amylase production was measured by determining the converting power of the mold filtrates when the latter were added to a solution of soluble starch. This in turn was correlated with test fermentations. No attempt was made to differentiate between dextrogenic and saccharolytic activity. Future references to the percentage of conversion refer to the amount of sugar produced from the starch expressed as the percentage of its total sugar equivalent. The details of the method used are given below.

#### METHOD FOR THE DETERMINATION OF FUNGAL AMYLASE ACTIVITY

This method is adapted for its present use from a combination of two analytical procedures from the A O A C, sixth edition (1945). The methods are 20.61, page 257, and 20.28, pages 244-245. Modifications are noted in the following description.

The following are placed in a 100-ml Erlenmeyer flask,  
20 ml starch solution (3 per cent Lintner's soluble starch solution)  
25 ml buffer solution (double strength of solution "a," p 244)  
1 ml fungal amylase (filtrate from mold culture)

Hold 1 hour at 30 C in a water bath. Add 2 ml 3.58 N H<sub>2</sub>SO<sub>4</sub> and 2 ml sodium tungstate (12 per cent solution). Filter at once, discard first 8 to 10 drops. Pipette a 5-ml aliquot into a 1-by-8-inch pyrex test tube and add 10-ml ferricyanide solution (p 244). Hold exactly 20 minutes in a boiling water bath. Cool to room temperature and empty the tube into a 100-ml Erlenmeyer flask. Rinse with 25 ml acetic salt solution, add 1 ml starch + KI solution, and titrate with 0.1 N thiosulfate solution. Record ml of thiosulfate used. Run a blank containing only starch and buffer in each test.

**Calculations.** Use table on page 245, A O A C, sixth edition, to find the amount of maltose corresponding to the 0.1 N ferricyanide used. Divide by 20



to give the mg of maltose per 60 mg of starch This figure divided by 60 gives the percentage of conversion in 1 hour at 30 C Example

Blank	9.68 ml
Test	1.52 ml
	<hr/>
	8.16 ml = $\frac{475}{20} = 23.75$ mg maltose
	$\frac{23.75}{60} = 39.58\%$ conversion

If the results show over 40 per cent conversion, a smaller aliquot than 5 ml may be taken for the ferricyanide test and the results calculated accordingly

#### EXPERIMENTS DIRECTED TOWARD INCREASING AMYLASE PRODUCTION BY *A. NIGER* NRRL337

The work reported here was undertaken with the idea of shortening the time required to develop fungal amylase of the desired strength. In order to obtain amylolytic preparations of the required potency, Le Mense, Corman, Van Lanen, and Langlykke (1947) aerated their cultures of *A. niger* for from 3 to 5 days. These cultures were developed in grain stillage supplemented with 2 per cent of corn meal and adjusted to pH 4.9 to 5.3 with calcium carbonate. Such a procedure would encounter difficulties in large-scale use owing to the long period of growth during which infections would be likely to develop.

Although the use of stillage is economically desirable in plant practice, in the interest of uniformity the basic medium employed in the laboratory experiments reported here had the following composition, A 10 per cent corn mash that had been acidified with sulfuric acid to pH 4.0 was cooked for 1 hour at 30 pounds steam pressure. The mash was then adjusted to the desired grain concentration and to a pH of 5.3 with calcium carbonate. It was then reesterilized in 100-ml amounts in 1,000-ml Erlenmeyer flasks. In the experiments reported 0.25 per cent urea was also added to all flasks, as this substance appears to stimulate the formation of dextrogenic enzymes.

It will be seen from the following experiments that the amount of amylase produced by *A. niger* NRRL337 will vary considerably with slight changes in the composition of the basic medium. For example, during investigations of the effects of adding small quantities of substances to the mold-growing medium it was noted that sodium or potassium chloride added in small amounts had a marked stimulating effect upon amylase production. It is of particular interest that the action of these chemicals appears to be culture-specific, for little or no response was obtained when they were added to cultures of "*Rhizopus boulard*." Appropriate experiments have shown that the salts actually stimulate production of amylase by *A. niger* and that we are not dealing with the known catalytic action of small amounts of salt on soluble-starch-amylase reactions.

The results in table 1 are typical of a number of experiments in which the addition of sodium or potassium chloride was shown to increase amylase production. One hundred ml of the basic medium previously described plus sodium

chloride as indicated were inoculated with spores of *A niger* NRRL337 and shaken continuously for 24 hours at 30 to 32 C on a shaking machine. The mold culture was then filtered through paper and the filtrate tested for starch-converting power.

As will be shown subsequently in the discussion of pilot plant fermentations, it is necessary to produce a mold amylase giving 20 per cent conversion of soluble starch under the conditions we have described in order to get satisfactory conversion for an alcoholic fermentation of corn. Amylolytic preparations of lower potency are correlated with low yields of ethyl alcohol when they are employed as the converting agent in grain fermentations. The use of small amounts of

TABLE 1

*The effect of NaCl and KCl additions to grain mashes on the production of amylase by Aspergillus niger NRRL337*

SALT CONCENTRATION	PER CENT CONVERSION
<i>g per 100 ml</i>	<i>24 hours</i>
0.001 NaCl	27.83
0.001 KCl	23.80
0.002 NaCl	26.13
0.002 KCl	22.50
Control	6.40

TABLE 2

*The effect of concentration of the growing media on the production of amylase by Aspergillus niger NRRL337*

CONCENTRATION	PER CENT CONVERSION		
	22 hr	42 hr	96 hr
<i>Degrees Brix</i>			
6	23.0	32.1	39.0
8	21.2	31.6	45.4
10	20.2	32.8	70.3

sodium chloride has, therefore, a practical value since its use makes possible the preparation of amylolytic solutions of the desired strength in shorter time.

Another factor influencing the production of amylase by the culture under study is the grain concentration of the growing medium. In table 2 there is shown the result of a series of mold shake flasks in which grain mash plus optimum amounts of sodium chloride were set up in three different concentrations. It will be seen that as grain concentration increased, enzyme production also increased. The flasks were prepared and shaken continuously as in the preceding experiment.

It can further be seen from table 2 that if it is desired to use the fungal amylase at 22 hours there is no advantage in raising the concentration above 6 Brix,

but, on the other hand, if there is no practical disadvantage in extending the growing period the converting power can be increased, in this instance three and one-half times, by raising the grain concentration to 10 Brix and growing for 96 hours

EFFECT OF ANTISEPTICS UPON AMYLASE PRODUCTION BY *A. NIGER*  
NRRL337

When one attempts to move a laboratory fermentation to pilot plant or full-scale commercial operation, contamination by undesirable microorganisms frequently interferes with the production of the desired products. It was found necessary to maintain submerged cultures of *A. niger* NRRL337 virtually free from bacterial contamination. Failure to do this interferes seriously with amylase production. In order to introduce a factor of safety into plant operation a number of laboratory tests were conducted to determine the conditions under which antiseptics might be employed. It was found possible to select

TABLE 3

*The adverse effect of bacterial contamination upon amylase production by Aspergillus niger NRRL337, and its control by ammonium bifluoride*

CONCENTRATION OF AMMONIUM BIFLUORIDE	PER CENT CONVERSION
<i>g per 100 ml medium</i>	<i>24 hours</i>
0.01	10.08
0.015	18.12
0.02	14.97
0.03	10.42
0.04	8.83
0.05	8.17
None	4.93

concentrations of either ammonium bifluoride or sodium pentachlorophenate ("dowicide G") which inhibit bacteria but allow mold growth and amylase production to proceed.

A typical example of this is shown in the following experiment in which a mash, contaminated with 0.05 per cent by volume of a young vegetative culture of *Clostridium acetobutylicum*, was seeded with mold spores and shaken for 24 hours. This organism was selected because, paradoxically enough, this anaerobe is able to grow symbiotically with an aerated mold culture, and anaerobic contaminants, either butyric or butylic, have been most commonly encountered.

Heavy contamination was observed microscopically in the culture to which no ammonium bifluoride was added. The flasks containing 0.02, 0.03, and 0.04 g per 100 ml ammonium bifluoride showed little bacterial growth, but the mold was inhibited. Flasks containing 0.015 and 0.02 g of ammonium bifluoride per 100 ml of mash showed the most favorable balance between mold activity with slight bacterial contamination and maximum amylase production.

Similar results have been obtained with dowicide G. However, this anti-

septic in addition to controlling bacterial contamination in submerged cultures of *A. niger* markedly inhibits spore formation. Untreated shake cultures occasionally developed spores on the sides of the glass flask above the liquid level, but in the presence of the proper amount of dowicide G spores were not formed. Since sporulation under these conditions usually was accompanied by poor amylase production, it was found possible to eliminate irregular results in laboratory shake cultures because of sporulation of mold by suitable dosage of the cultures with dowicide G.

The following is typical of a number of experiments in which it was noted that (1) sporulation in shake flasks is associated with low amylase production, and (2) the correct amount of dowicide G inhibits sporulation and does not interfere with amylase production.

The flask containing no antiseptic showed marked sporulation. Amylase production was fair at 24 hours but a large part of the enzyme was destroyed, possibly by products associated with sporulation, when shaking was continued up to 48 hours. Flasks 3 and 4 showed slight or no sporulation and the highest

TABLE 4

*Effect of dowicide G on sporulation and amylase production by Aspergillus niger NRRL337*

NO	DOWICIDE G ADDED <i>g per 100 ml mash</i>	PER CENT CONVERSION		SPORULATION
		24 hr	48 hr	
1	None	23.8	5.81	Heavy
2	0.0012	43.7	23.8	Slight
3	0.006	46.6	43.7	Slight
4	0.012	46.6	46.6	None
5	0.024	15.2	15.2	—

amylolytic activity. Flask 5 showed inhibition of both mold growth and amylase production because of excess of dowicide G. It is not known how general this inhibitive action of dowicide G upon mold sporulation may be, although it has been observed in experiments in this laboratory in the case of a number of mold strains.

By supplementing the basic medium with optimum amounts of sodium chloride and dowicide G, laboratory shake cultures seeded with *A. niger* spores developed the minimum required potency of 20 per cent conversion within 24 hours. Laboratory fermentation tests employing fungal amylase, upon which the pilot plant runs subsequently described were based, followed the general plan given below.

Eighty-four g corn meal, 500 ml water, and 0.84 g barley malt (1 per cent premalt) were heated to 66 C, with frequent hand shaking. The flasks were then autoclaved 1 hour at 20 pounds steam pressure and cooled to 60 C, and the desired amount of amylase was added. Conversion was allowed to proceed at 60 C for 1 hour, and the flasks were cooled to 30 C and seeded with 4 per cent

by volume of a suitable yeast Under these conditions the yields given in table 5 were obtained

#### PILOT PLANT FERMENTATIONS

In table 6 are given pilot plant yields obtained from whole ground corn when fungal amylase was employed as the converting agent The general procedure followed in these fermentations was as follows

Corn mash acidified to pH 4.0 and cooked by the continuous high-pressure steam jet method (Unger *et al.*, 1944) was cooled, diluted to 6 to 8 Brix, and neutralized to pH 5.3 with calcium carbonate Urea was added at the rate of 2 pounds per 1,000 gallons of mash and sodium chloride at the rate of 25 g per 1,000 gallons of mash The mash was then sterilized at 40 pounds steam pressure for 1 hour, cooled, and 200 gallons were inoculated with a saline suspension of *A. niger* spores, prepared by suspending the spores grown on a heavy corn mash pad in a 250-ml Erlenmeyer flask in 2,000 ml of 0.8 per cent saline solution

TABLE 5

*Ethyl alcohol yields from corn meal converted by Aspergillus niger NRRL337 fungal amylase*

NO	FUNGAL AMYLASE	MALT	FERMENTATION EFFICIENCY	LABORATORY YIELD* ETHYL ALCOHOL
	vol %	per cent	per cent	proof gallons
1	10	None	85.7	5.56
2	20	None	92.0	6.03
3	10	2	89.6	5.80
4	20	4	91.5	5.90
5	20	2	94.0	6.09
6	20	4	95.7	6.29

\* Laboratory yield expressed as proof gallons of ethyl alcohol per bushel of grain, wet basis

The inoculated tank was aerated continuously, and at 48 hours its amylase content usually tested the required 20 per cent conversion This period may be shortened by introducing an intermediate vegetative mold stage Grain stillage supplemented with 2 per cent corn meal and with urea and sodium chloride has also been used with success The latter medium is more economical than corn mash, but it is somewhat less uniform because of variation in the stillage The final or fermenter stage was made up in a 1,000-gallon tank equipped with an agitator Six hundred gallons of corn mash (1,120 pounds of corn meal plus 1 per cent premalt) were premalted in the conventional manner, after which the thinned mash was cooked at 35 pounds steam pressure for 1 hour It was then cooled to 60 C and 10 per cent by volume of fungal amylase added As soon as the mold had been added to the 600 gallons of corn mash, the temperature was brought down by means of cooling coils to 30 to 32 C and 2 per cent by volume of yeast was added The yields of ethyl alcohol obtained by this procedure are given in table 6 In considering these figures one should recall that

approximately 50 proof gallons of ethyl alcohol per bushel of whole ground corn of 12 per cent moisture is a normal plant yield when the fermentation is conducted by conventional malting procedures

It will be noted that the runs shown in table 6 were made with amylase preparations averaging 34.87 per cent conversion, and the yield, as would be expected from previous laboratory tests, was good, 5.61 proof gallons per bushel. A series of 6 runs previously made using amylase preparations averaging 7.32 per cent conversion gave only 4.30 proof gallons per bushel. This illustrates the correlation between the conversion power as shown by the test described and the alcohol produced. The 6 low-yielding pilot plant runs to which we have referred were in all cases due to poor amylolytic preparations. When conditions contributing to contamination were eliminated and when the mold-growing medium was properly modified along the lines described here, amylase produc-

TABLE 6

*Fermentation efficiency—seven 600 gallon pilot plant runs—employing fungal amylase averaging 34.87 per cent conversion*

RUN	FERMENTATION EFFICIENCY	ETHYL ALCOHOL* PROOF GALLONS
	<i>per cent</i>	<i>per bushel</i>
1	90.7	5.65
2	92.6	5.95
3	91.1	5.86
4	90.9	5.90
5	84.5	5.50
6	83.1	5.34
7	78.6	5.06
Average	87.4	5.61

\* Yield expressed as proof gallons of ethyl ethyl per bushel of grain, wet basis

tion and ethyl alcohol yields comparable to those in table 6 were invariably obtained

Although we do not know at present that sporulation of *Aspergillus niger* such as we have described in shake flasks occurs in a plant vessel that is being aerated, we have found that the use of up to 0.01 g per 100 ml of dowicide G has no harmful effect upon mold growth and introduces a desirable safety factor into plant operation by its inhibitive action on bacterial contaminants

#### SUMMARY

Laboratory and pilot plant fermentations confirm the observations of the Northern Regional Research Laboratory workers on the efficiency of *Aspergillus niger*, strain NRRL337, as a converting agent for grain fermentations

Amylase production by this culture is stimulated by the addition of small quantities of NaCl or KCl to the growing medium

In order to get a satisfactory alcohol yield, a minimum of 20 per cent conversion

by the test described is required when 10 per cent by volume of fungal amylase is employed in grain fermentations

Yields of from 60 to 63 proof gallons of ethyl alcohol per bushel of wet corn have been obtained in the laboratory, and 56 proof gallons of alcohol, on the same basis, have been obtained in pilot plant fermentations using fungal amylase produced as described

Sodium pentachlorophenate and ammonium bifuoride may be employed to control bacterial contamination in fungal amylase production by *A. niger* NRRL-337. The inhibitive action of sodium pentachlorophenate on the sporulation of *A. niger* in shake flasks has been noted. Sporulation in laboratory shake flasks is frequently correlated with low amylase production, and consequent low alcohol yield in the subsequent yeast fermentation of the converted grain mash

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# STUDIES WITH THE ELECTRON MICROSCOPE ON THE INTERACTION OF RED CELLS AND INFLUENZA VIRUS

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The influenza virus, red cell agglutination phenomenon, first observed by Hust (1941) and McCellind and Hue (1941), has proved to be a reaction of considerable importance. In addition to its practical value this phenomenon offers an opportunity to study a virus and cell interaction of general theoretical interest. It is generally conceded that adsorption of virus on the susceptible cell surface constitutes the first step in the process of infection by a virus. The adsorption of influenza virus on red cells has been amply demonstrated by indirect methods and strengthens the analogy between the virus-red-cell reaction and the initial phase of virus infection (Hust, 1942, Francis and Salk, 1942). It is the purpose of this paper to present direct evidence of the interaction between virus and red cell.

In work with multicomponent biological materials the electron microscope technique presents considerable difficulties. For example, in the demonstration of an antigen-antibody reaction, for which relatively high concentrations of materials may be needed, the problem of clearing the specimen background arises in obtaining electron micrographs of satisfactory quality. With such a system it is necessary that care be exercised in the washing process for clearing the supporting film, in order that the desired features of the preparation may not be lost.

Even more difficult to study with the electron microscope is the interaction between proteins and cells. Here osmotic equilibrium must be maintained, since the removal of salt may result in cell destruction. This problem is found with systems involving human red cells, as hemolysis is initiated easily and the subsequent liberation of inner structural material alters the experimental conditions. In such cases it is easier to work with cell ghosts, provided that they still retain the desired interacting properties. In general, with a knowledge of the difficulties involved and a suitable handling of the system, satisfactory electron micrographs can be obtained at various stages of virus and red cell interaction.

## MATERIALS

Virus samples were obtained from chick allantoic fluid and purified by the use of the Sharples centrifuge. Both active influenza virus type B and formalin-inactivated A and B mixtures were employed in the experiments.

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\* I am indebted for virus samples to Dr. B. Hampil of the Sharpe and Dohme Company, Inc.

Human red cell ghosts were prepared by the following procedure: (1) Washed cells were hemolyzed in distilled water for 30 minutes and sedimented by low speed centrifuging. (2) The sedimented cells were washed twice in saline. (3) The final sediment was stored at 5°C in saline.

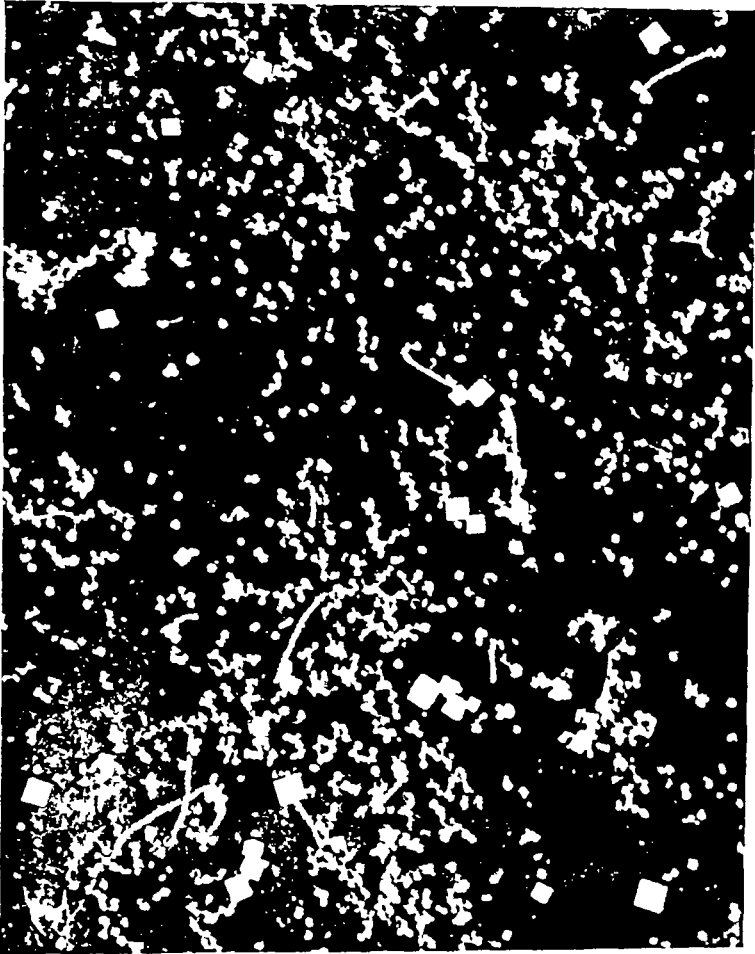


FIG. 1. INFLUENZA VIRUS A AND B VACCINE MIXTURE, DEMONSTRATING VARIOUS STAGES OF AGGREGATION AND DISINTEGRATION OF PARTICLES.

The length of the scale is 1 micron.

Washed chicken red cells can be used directly, since they are less easily hemolyzed during the washing of the specimen. The RCA type B electron microscope, without objective aperture, was used.

#### METHODS

Electron microscope specimens were prepared in the following manner: (1) Virus solution was diluted in distilled water and a drop of the suspension was placed on the specimen screen. After a few minutes the excess fluid was re-

moved and the specimen screen dried. The screen was then dipped several times in distilled water and, after again drying, gold shadow casting was performed (Williams and Wyckoff, 1945a). The same technique was also used for human red cell ghosts. Intact human and chick red cells were suspended in saline and handled in the usual manner. The human red cells were very sensitive to hemolysis, and the single dipping of the specimen into water for the removal

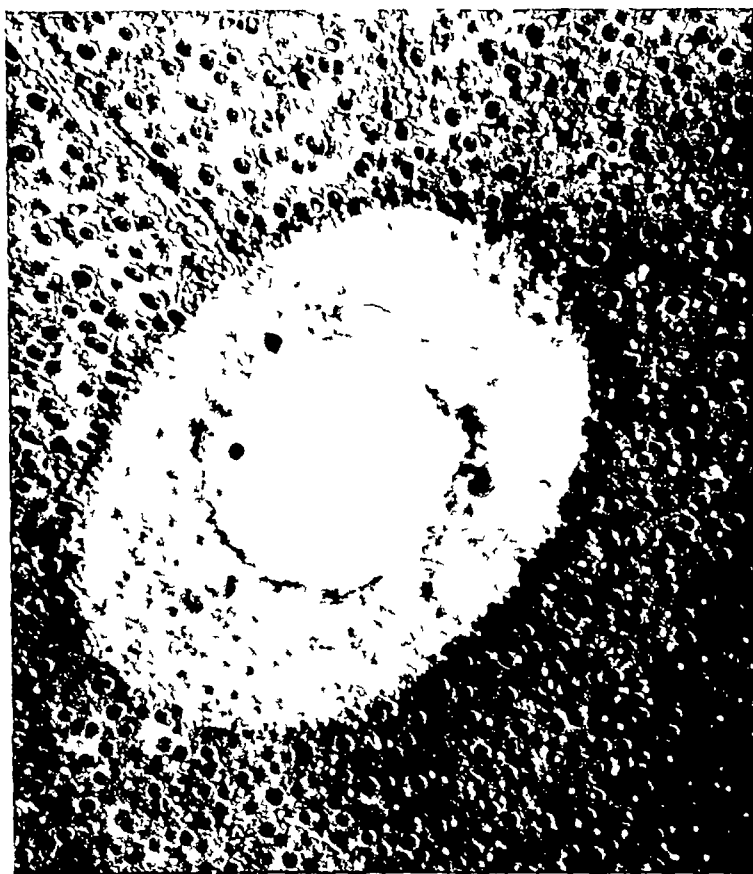


FIG. 2. INFLUENZA B VIRUS ADSORBED ON CHICK RED CELL AFTER 12 MINUTES IN CONTACT IN SALINE SOLUTION (TEMP. 6°C)

The craters on the film are due to defects in the supporting film. The length of the scale is 1 micron.

of salt caused partial hemolysis. (2) Virus and cells (or red cell ghosts) were mixed and kept in contact at a constant temperature for the required time. The suspension was then diluted in saline, or in distilled water, and one drop was placed immediately on the specimen screen and handled in the previously described manner. The washing has to be done carefully in this case, otherwise the cells are easily washed off the screen.

## EXPERIMENTAL RESULTS

*Virus*—The influenza virus used for interaction experiments appeared in the electron microscope to be in the same particle size range as estimated from electron microscopic data by Fiktor *et al.* (1943), Williams and Wyckoff (1945b) and others, the particles being round and of diameters ranging from 75 to 130  $m\mu$ . It was observed that during storage in 0.9 per cent saline at 5°C. inactivated virus started to change its configuration and a variety of shapes appeared sug-

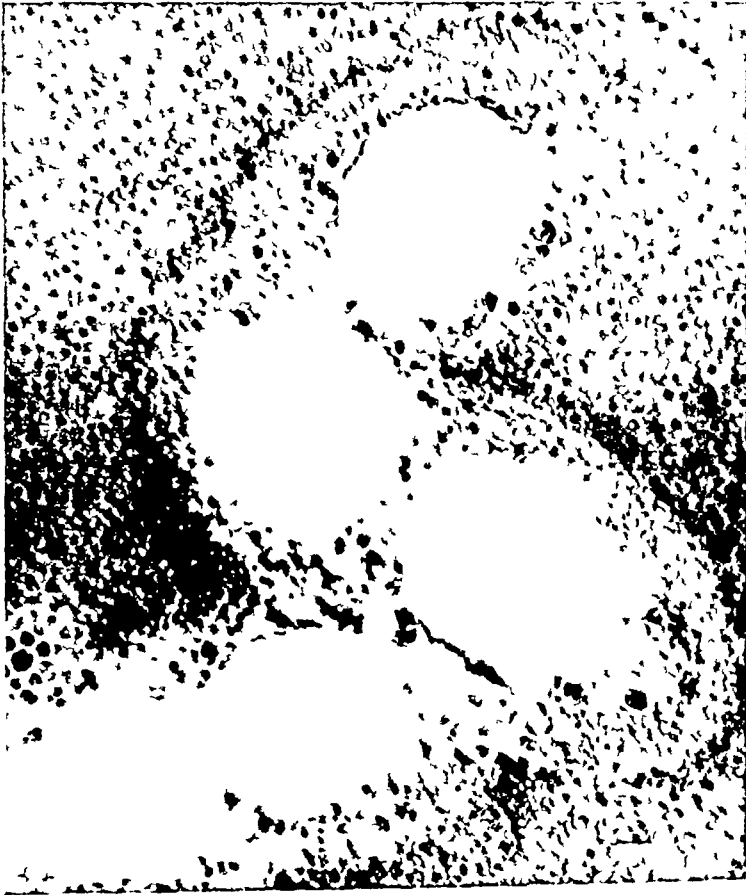


FIG. 3. CHICK RED CELL ACCRETION IN THE PRESENCE OF INFLUENZA B VIRUS.  
SAME SAMPLE AS FIGURE 2 WITH 15 MINUTE CONTACT.

The length of the scale is 1 micron.

nea, are also present. It has been observed from many other similar micrographs that disintegration of particles into smaller fragments takes place when the surface area has increased too extensively. Generally there are many modes of disintegration and aggregation. White rectangular particles on the micrographs are magnesium oxide crystals, introduced on the specimen in the form



FIG. 4. INFLUENZA VACCINE A AND B MIXTURE, ADSORBED ON HUMAN RED CELL GHOST AFTER 20 HOURS' CONTACT AT 4°C

The length of the scale is 1 micron

of smoke. They were used for determining the height of the particles. This subject will be discussed elsewhere.

In the most orderly instances of aggregation particles seem to form filaments of various lengths. The examination of samples stored for longer periods of time showed further increase of filamentous particle forms and general loss of definition of individual particles.

*Virus and chick red cells.* Figure 2 shows a chick cell with a dense nuclear area and a relatively less dense peripheral region. This outer region is sufficiently

transparent for virus interaction studies. Virus is seen to be attached to the border area of the cell and to the surface of certain areas of the less dense cell region. Other areas of the cell surface are too dense for the resolution of individual virus particles.

The fibrous material seen on one side of the cell also has some virus particles attached. The background criteria are defects of the supporting film. Figure 5



FIG. 5. AGGLUTINATION OF HUMAN RED CELL GHOSTS BY INFLUENZA A AND B VACCINE

The length of the scale is 1 micron

is a micrograph of chicken red cells agglutinated by influenza virus, again demonstrating the surface adsorption of the virus particles.

*Virus and human red cells.* The adsorption of inactivated virus on the ghost surface is shown in figure 4, where virus particles and filaments of various lengths cover both sides of the ghost. Particles on the upper surface of the cell are more clearly defined and are not covered by the cell wall. Figure 5 demonstrates the agglutination of ghosts by virus.

Surface saturation of a ghost cell by active virus is shown in figure 6, the screen having been prepared by adding relatively few ghosts to a concentrated

virus solution. As can be seen, the average concentration of virus on the ghost surface is much larger than that on the background film.

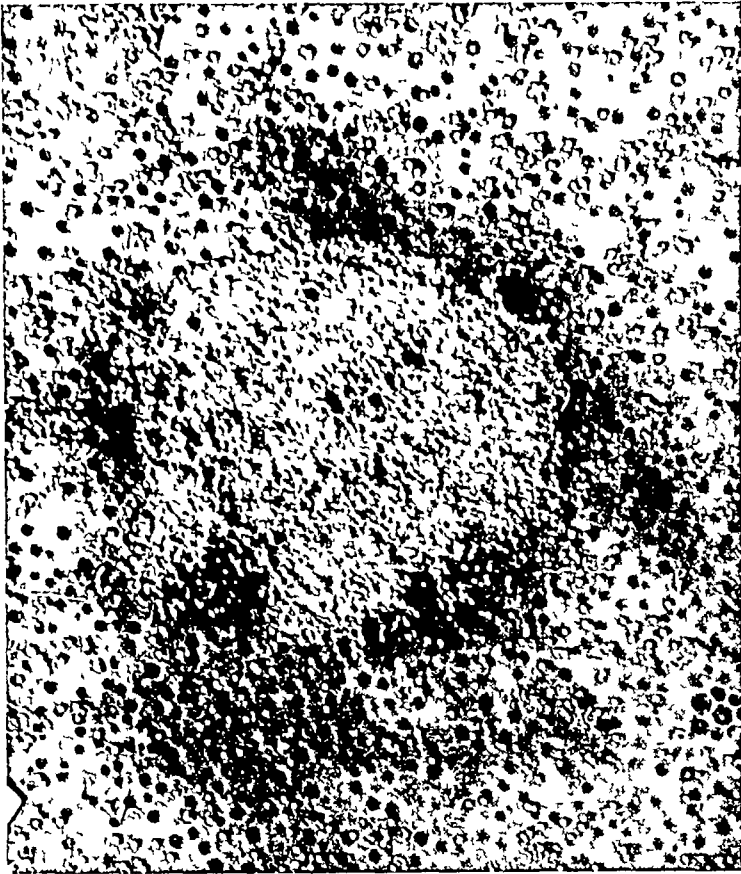


FIG. 6. INFLUENZA B VIRUS ADSORBED ON HUMAN RED CELL GHOST, CONTACT 30 MINUTES AT 4 C, EXCESS VIRUS.

The length of the scale is 1 micron.

#### DISCUSSION

It is of interest to know how the immunochemical properties of a virus particle are affected when a change takes place in its external geometry. It is expected that some information concerning structure can be gained by studying thoroughly the modes of deformation. At the present time, the experimental data are insufficient for such an analysis, but it can be stated that in some cases of aggregation the interacting properties of this virus are not destroyed. As the evidence presented shows, filament type virus particles can still adsorb on the red cell surface. It is uncertain at what stage of disintegration the virus particle fractions become incapable of interacting with cell surfaces. Fractionation

studies, accompanied by chemical experiments, might offer an opportunity to locate the biologically active groups of virus particles.

It is uncertain whether agglutination of red cells is caused by the virus particles directly, forming a linkage between agglutinated cells, or whether the cell surfaces are sensitized by adsorbed virus and subsequently agglutinated. Repeated observations with the aid of the electron microscope indicate that fresh, unstored red cells, when agglutinated by virus, always show its presence on the surface and especially on the contact area of the cells. As revealed by figure 6, a human red cell ghost is also capable of adsorbing virus over practically all of its surface. The observations further indicate that certain red cells that are not agglutinated may still have virus adsorbed on their surfaces. This is usually the case at relatively low virus concentrations. The observation suggests that virus adsorption does not sensitize the cell surface for agglutination, but rather that the most probable explanation, based on a large number of observations, is that the agglutinating action of influenza virus on the red cells is a direct one. The phenomenon appears to take place, however, only when there is a sufficient number of virus particles present to form linkages between the cells. Furthermore, any visible precipitation of red cells indicates rather an extreme case of interaction, since combination between virus and cells can take place without producing any visible agglutination by a standard test-tube technique.

Electron microscopic studies have so far not revealed any visible changes on the red cell ghost surface after the virus elution, but further studies should be done on the subject. The difficulties are considerable because the large number of holes in the cell wall and the extensive disorganization of the surface, even before virus adsorption, make changes difficult to detect. On the other hand, the normal red cell, with its intact cell wall, which would be more suitable in that respect, is too dense for study with the electron microscope. Replica studies might be helpful.

#### ACKNOWLEDGMENTS

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#### SUMMARY

Electron microscopic studies suggest that inactive influenza virus undergoes deformation into various forms during prolonged storage. Some aggregation forms, at least, appear to retain the property of interaction with red cells.

Electron micrographs are presented showing the physical relationships between influenza virus and agglutinated red cells or red cell ghosts.

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# THE EFFECT OF SULFATHIAZOLE ON THE RATE OF INCREASE OF RIBOFLAVIN PRODUCTION BY *PROTEUS VULGARIS* AND *BACILLUS SUBTILIS*

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In recent years, sulfonamides have become very important in the chemotherapeutic treatment of various bacterial diseases. For this reason, it is desirable to understand more clearly their mode of action and their effect on human nutrition. One method of approaching both of these problems is by studying the effect of chemotherapeutic agents on the synthesis of growth factors essential to bacterial nutrition. Although a number of theories have been suggested regarding the mode of action of sulfonamides (Woods and Fildes, 1940, Henry, 1943, Sevag and Green, 1944), there are still many factors that have not been explained to complete satisfaction. One of these factors is the effect of sulfathiazole on riboflavin production. This paper is concerned with the synthesis of riboflavin by two nonpathogenic organisms that are sensitive to sulfathiazole and that synthesize relatively high amounts of riboflavin. Since riboflavin is an important factor in bacterial respiration, more light may be shed on this complex problem of sulfonamide action by observing the effect of this drug on riboflavin synthesis.

When bacterial cells synthesize riboflavin, they not only produce sufficient quantities for their own metabolism, but also release an excess into the substrate. According to Burkholder and McVeigh (1942), this amounts to more than 15 per cent of the total quantity synthesized in 48 hours. Therefore, by determining the amount that is present in the culture medium and cells at various time periods, the rate of increase of riboflavin and the effect of sulfathiazole on this increase can be determined.

## EXPERIMENTAL PROCEDURE AND RESULTS

Two species of bacteria taken from the culture collection of the Bacteriology Department of the University of North Carolina were used as the test organisms *Proteus vulgaris*, no. 355, and *Bacillus subtilis*, no. 480.

For the cultivation of *P. vulgaris*, the synthetic medium described by Burkholder and McVeigh (1942) was used as the medium of choice. When *B. subtilis* was used as the test organism, a medium described by Games and Stahly (1943) for *Leuconostoc mesenteroides* was used. In the latter case, the B complex vitamins that were not necessary for growth were omitted and 25 mg of asparagine per 100 ml of medium were added. In each experiment the inoculum consisted of cells from a 24-hour culture of the test organism that had been grown in the predescribed medium. The cells were washed twice by centrifugation and resuspension in 0.85 per cent NaCl solution.

In each series of experiments two inoculated culture flasks were used flask no 1 contained 1 mg of sulfathiazole per 100 ml of medium, and flask no 2, which served as the control, contained no sulfathiazole. The first series was incubated in a 30 C water bath and the second series in a 37 C water bath. Samples were removed from each flask at 2-hour intervals for 6 hours, then again at 24 and 48 hours for plate counts and riboflavin assays. Riboflavin assays were made

TABLE 1

*The effect of sulfathiazole on the synthesis of riboflavin by Proteus vulgaris at 37 C*

TIME HOURS	NO OF CELLS/ML	LOG NO OF CELLS/ML	TOTAL RIBOFLAVIN PRESENT, $\mu\text{G/ML}$	RATE OF INCREASE* OF RIBO / $1 \times 10^7$ CELLS/HR $\times 10^4$
Control—Absence of sulfathiazole				
0	15,000,000	7 18	0 0062	-5 0†
2	17,000,000	7 23	0 0045	
4	30,000,000	7 47	0 0065	3 3
6	156,000,000	8 19	0 015	2 7
25	370,000,000	8 57	0 145	1 8
48	2,300,000,000	9 36	0 32	0 3
Presence of sulfathiazole				
0	12,800,000	7 10	0 0013	
2	14,000,000	7 15	0 0058	16 0
4	17,000,000	7 28	0 0072	18 0
6	28,700,000	7 46	0 0097	4 4
25	110,000,000	8 04	0 0353	1 2
48	204,000,000	8 31	0 068	0 6

\* Difference between the rate of synthesis and utilization (see Discussion)

$$\dagger \text{Rate of increase} = \frac{\text{Increase (positive or negative) in total riboflavin}}{\text{Total no of viable organisms} \times \frac{1}{10^7}} \times \frac{1}{\text{Time interval in hours}} \times \frac{1}{10^4}$$

An example for the table above

$$\text{Rate of increase} = \frac{0.0045 (-0.0062)}{17,000,000 \times \frac{1}{10^7}} \times \frac{1}{2} = -0.00050 \times \frac{1}{10^{-4}} = -5.0$$

according to the method described by Snell and Strong (1939), using *Lactobacillus casei* as the test organism. The culture flasks and assay samples were protected from light at all times to prevent inactivation of the riboflavin.

The results obtained when *P. vulgaris* was used as the test organism and incubated at 37 C are recorded in table 1 and shown graphically in figure 1. Sulfathiazole was added in concentrations of 1 mg per 100 ml of medium. In

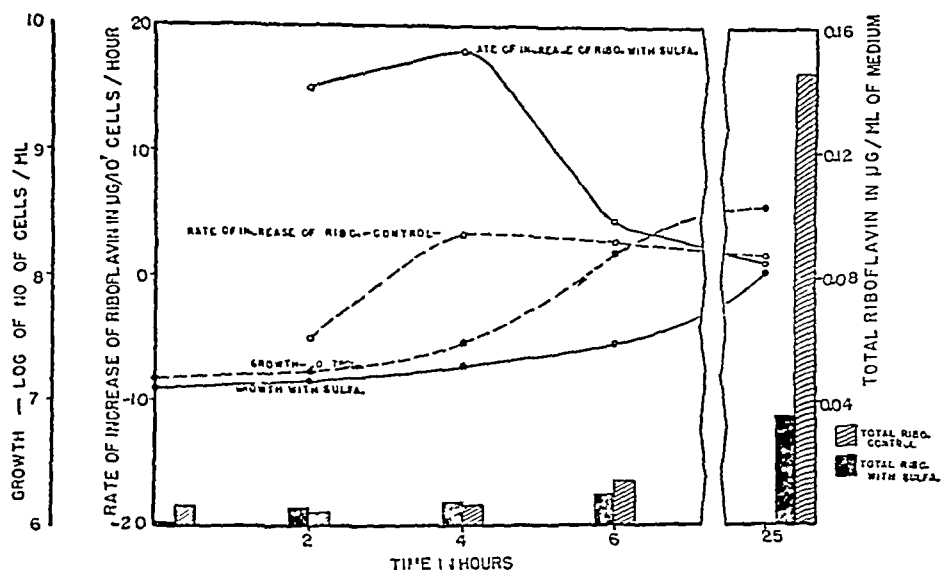


FIG 1 THE RELATIONSHIP OF THE RATE OF INCREASE OF RIBOFLAVIN, THE TOTAL RIBOFLAVIN CONTENT, AND THE GROWTH RATE OF *P. VULGARIS* IN THE PRESENCE AND ABSENCE OF SULFATHIAZOLE AT 37 C

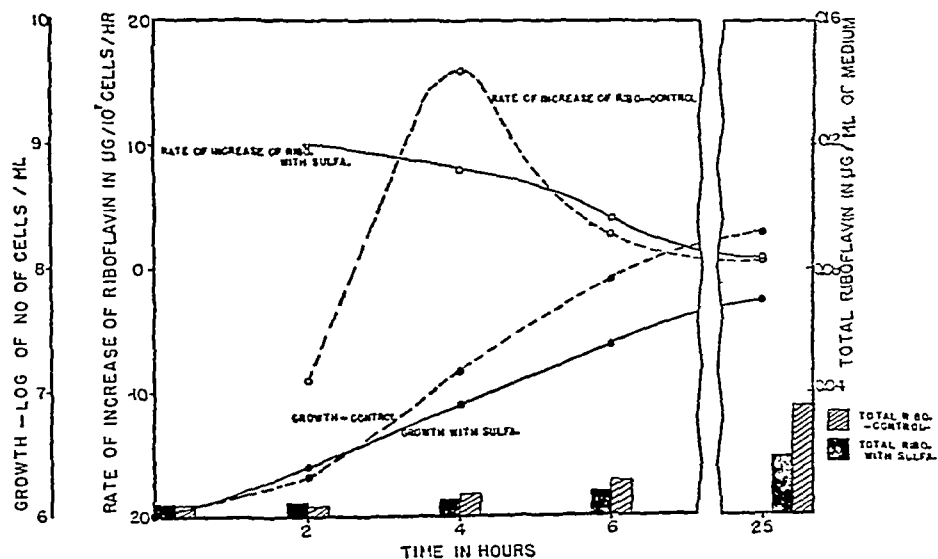


FIG 2 THE RELATIONSHIP OF THE RATE OF INCREASE OF RIBOFLAVIN, THE TOTAL RIBOFLAVIN CONTENT, AND THE GROWTH RATE OF *B. SUBTILIS* IN THE PRESENCE AND ABSENCE OF SULFATHIAZOLE AT 37 C

each case a control experiment was conducted under similar conditions, omitting the sulfathiazole from the medium. The rate of increase of the riboflavin was

highest when the medium contained sulfathiazole. Furthermore, the highest rate of increase occurred in the earlier phases of the cultural growth. When the culture flasks were incubated at 30 C, the results obtained were similar to those shown in table 1. The higher rate of increase of riboflavin occurred in the medium containing sulfathiazole, and the greatest amount of riboflavin was synthesized in the earlier phases of the cultural growth.

In order to compare the effect of sulfathiazole on another strain of bacteria, *B. subtilis* was used as the test organism. The results obtained from these experiments are shown in figure 2. Although the results are similar to those obtained for *P. vulgaris* for the first 3 hours, the rate of increase is not so high in the presence of sulfathiazole. This is probably due to the fact that the cells are not so strongly inhibited by the drug in this experiment as in the previous one. This can be seen by examining the growth rate of these cells. The total riboflavin per ml of medium is also lower with *B. subtilis*, but this is due, undoubtedly, to the lower cell count at each time interval. However, the same general trend is evident with this organism as with *P. vulgaris*, there is a higher rate of increase of riboflavin when sulfathiazole is present in the medium.

#### DISCUSSION

The total amount of riboflavin present at the zero time period is assumed to be due to the riboflavin present in the washed bacterial cells, since the medium was riboflavin-free. It is not possible to assume that the riboflavin present at the zero time period was utilized completely before synthesis began, therefore, the rate of synthesis cannot be measured directly. However, the amount of riboflavin synthesized in excess of the amount utilized can be measured and is designated in this paper as the rate of increase of riboflavin. As the values for the rate of increase of riboflavin that would be obtained on a per-cell basis are so small, the results in this paper are expressed on a per-10,000,000-cell basis.

The rate of increase of riboflavin was found to be highest during the lag phase. This was to be expected as the period of "physiological youth" occurs during this phase and the higher rate of metabolic activity going on at this time would cause a subsequent increase in the amount of riboflavin present.

The total amounts of riboflavin found present at each time interval are not too significant by themselves. However, when they are converted to a rate of increase expressed in terms of 10,000,000 cells per hour and compared over a period of 48 hours, they indicate the general trend. In each case there is a definite indication that the highest rate of increase of riboflavin is during the lag phase, and that a higher rate of increase occurs when sulfathiazole is present in the medium in concentrations of 1 mg per 100 ml of medium.

There appear to be at least two possible explanations for this higher rate of increase of riboflavin occurring when sulfathiazole is present in the culture medium. This increase may be due to a stimulating effect by this concentration of the drug on the enzyme system responsible for riboflavin synthesis, or to the drug's preventing the bacterial cell from utilizing the riboflavin. As the enzyme system concerned with riboflavin synthesis is one of a chain of enzyme systems

involved in bacterial respiration, an inactivation of one of these enzyme systems by the drug would inhibit the utilization of a product of another system in the chain. This would cause a higher concentration of the product to be present in the cell and a subsequent increase in its amount in the substrate. As the processes involved are of a very complicated nature, it is rather difficult to ascertain at this time which, if any, of these theories are correct.

#### SUMMARY

When *Proteus vulgaris* and *Bacillus subtilis* are grown in synthetic media, the highest rate of increase of riboflavin occurs during the lag phase of growth.

When sulfathiazole is added to the medium in concentrations of 1 mg per 100 ml of medium, a higher rate of increase of riboflavin occurs during the first 6 hours of growth.

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# THREE NEW SPECIES OF THE GENUS CLOSTRIDIUM

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At the last meeting of our society in Philadelphia a brief description was presented of three interesting species of the genus *Clostridium* (Spray, 1947) that had apparently not been previously identified. To these were assigned names presumed to be suitable to their morphologic and physiologic characters. Time did not permit their full description, and it is the intention to give here more detail, following the form used in *Bergey's Manual of Determinative Bacteriology*. In addition to the routine description certain reactions reported by the author (Spray, 1936, 1937) are included.

These three species were isolated from the sources indicated below, by methods given in the previously mentioned publications, including various procedures of enrichment, with subsequent use of the anaerobic dish proposed by the author (Spray, 1930).

## CLOSTRIDIUM NAUSEUM N SP

This species was isolated thrice from topsoil of the University campus. Soil samples were shaken in tubes of sterile tap water and heated for 10 minutes at 80 C, then inoculated into freshly boiled and cooled tubes of Difco brain liver heart (semisolid) medium covered with a heavy oil. After some 48 hours' incubation at 37 C several tubes yielded a most nauseating odor, far beyond that of ordinary putrefaction.

Stains from these cultures revealed several morphologic types. The cultures were then plated in Difco liver veal agar and incubated at 37 C in the anaerobic dishes. The same nauseous odor was observed upon opening several dishes, and a variety of well-isolated colonies were fished, with the aid of a wide-field binocular, to the semisolid medium under oil seal. Microscopic and cultural studies later revealed some of these cultures to be *Clostridium perfringens* and *Clostridium tertium*, but several fished from minute, lenticular, creamy colonies proved to be the organism in question, beyond doubt, from its characteristic odor.

*C. nauseum* n sp. Rods, 0.8 to 1.1 by 6.0 to 12.0 microns, with rounded ends, occurring singly, in pairs, and in short chains of 4 to 6 cells. Actively motile, especially in young cultures in semisolid medium, with numerous peritrichous flagella. Spores ellipsoid to elongate, subterminal, distinctly swelling the rods, often becoming apparently terminal at maturation. Gram-positive in early vegetative stage, but gram-negative at sporulation.

Gelatin (or iron-gelatin). Very slowly liquefied, softened at 14 days, completely liquefied at 30 days, not blackened even in the presence of an iron strip.

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Agar surface colonies (anaerobic) Minute, transparent, flat, slightly lobate

Agar deep colonies Minute, lenticular, entire, whitish to creamy

Blood agar not hemolyzed

Milk (with iron strip) Solidly coagulated at 4 to 5 days, clot shrinks slowly, but without gas, blackening, or digestion Evidently a rennet curdling, since the whey reaction was neutral to litmus

Indole Questionably formed, if so, obscured by an abundance of skatol Mercaptan is formed, together with other aromatic, putrid nitrogenous compounds not yet identified

Acid and gas from glucose, fructose, and maltose Sucrose, lactose, inulin, mannitol, sorbitol, glycerol, and inositol are not fermented

Nitrates are not reduced to nitrites

Coagulated albumin not liquefied

Blood serum not liquefied

Brain medium (Hibler) Blackened but not visibly digested

Lead acetate agar or peptone iron agar (Difco) Blackened in 24 hours

Nonpathogenic for white mice, guinea pigs, and rabbits

Optimum temperature Not determined, but grows well at both 37 C and at room temperature

Outstanding character The extremely nauseous, fecal odor, due apparently to some presently unidentified aromatic nitrogenous compound

Strictly anaerobic

Source Thrice isolated from soil

Habitat Not known other than in soil

#### CLOSTRIDIUM MICROSPORUM N SP

A tiny navicular organism isolated with considerable difficulty from a fatal case of peritonitis The peritoneal fluid, at autopsy, was peculiarly foul Stains showed the usual mixed flora, but also a few tiny navicular rods with sharply pointed ends and tiny spherical, central to eccentric, spores that slightly swelled the rods Spores of other anaerobes were more abundant, together with the usual mixed nonsporulating aerobic flora By selective heating the latter was eliminated, leaving apparently only three forms, later identified as *C. perfringens*, *C. tertium*, and the tiny navicular organism in question By passage through milk (followed by heating) *C. perfringens* was eliminated After plating the milk and incubating it at 37 C for 72 hours, tiny pin-point colonies were observed among the larger colonies of *C. tertium*, and finally after some 32 days' effort pure cultures of the desired organism were isolated

*C. macrosporum* n sp Rods, 0.8 by 2.0 to 4.0 microns, occasional long, pleomorphic filaments, distinctly vacuolate, especially in old cultures, occurring singly and in pairs but not in long chains Organisms navicular and sharply pointed at both ends Spores tiny, spherical, central to slightly eccentric, and slightly swelling the rods Actively motile particularly by a spinning movement with little progressive motion Presence, number, or position of flagella not detected

Gelatin (or iron-gelatin) Not liquefied nor blackened

Agar surface colonies (anaerobic) Tiny, almost imperceptible, transparent dewdrop colonies, very slightly raised, with entire edge, visible only after some 48 hours' incubation

Agar deep colonies Tiny, 0.5 to 1.0 mm, lenticular, with smooth entire edges, whitish-translucent (smaller and less opaque than *C. tertium*) Growth perceptible only after some 72 hours' incubation

Blood agar not hemolyzed

Milk (with iron strip) Fine and constant evolution of gas bubbles for many days, but no coagulation after 22 days' incubation Medium slowly grayed but not blackened

Indole is not formed

Acid and gas from glucose, maltose, and galactose Lactose, trehalose, rhamnose, raffinose, dulcitol, and inositol are not fermented

Nitrates are not reduced to nitrites

Coagulated albumin not liquefied

Blood serum not liquefied

Brain medium (Hibler) Not blackened nor digested, even in the presence of an iron strip

Lead acetate agar or peptone iron agar Not blackened

Nonpathogenic for white mice, guinea pigs, and rabbits

Optimum temperature Not determined, but grows better at 37 C than at room temperature

Outstanding characters The minute size, navicular pointed form, and the tiny spherical, central to eccentric spore

Strictly anaerobic

Source Isolated only once from the abdominal contents of a fatal case of peritonitis

Habitat Not known other than this single source

#### CLOSTRIDIUM GUMMOSUM N. SP.

A large, blunt rod, evidently a member of the "butyric group" from its later description. It was isolated without great difficulty, once from a case of gaseous gangrene, once from adult normal human feces, and once from feces of a normal infant of some 9 months' age. The colonies simulated those of *C. perfringens*, but further study quickly revealed active motility, abundant and early sporulation, and the absence of a capsule. Its fermentation pattern soon distinguished it as differing entirely from that of *C. perfringens*, nor did this pattern check with that of any of the described species of the "butyric" anaerobes.

It did display the typical "stormy fermentation," especially in iron-milk (Spray), but so also do many other of the "butyrics," particularly when heavily inoculated. However, it was soon observed that both the surface and, especially, the subsurface colonies were extremely mucoid in contrast to those of other members of the group. On the basis of these observations of the markedly gummatous character, and the failure of the fermentation pattern to fit into that of any known species, it is presented as

*C. gummosum* n. sp. Large rods, 0.8 to 1.0 by 4.0 to 8.0 microns, single and in

pairs, not in chains Sporulation active at 24 to 48 hours, spores eccentric to chiefly subterminal, large ovoid to elongate, markedly swelling the rods Moderate motility, increasing in activity up to 48 hours

Gelatin (or iron-gelatin) Not liquefied nor blackened at 19 days

Agar surface colonies (anaerobic) Large, round, convex, edge entire, very glistening and mucoid

Agar deep colonies Large, lenticular to buckwheat (*C. perfringens* type) White to creamy, very viscid to rubbery mucoid, entire colony dissected from the medium, or dragged unbroken by needle through 2 per cent agar (subsurface colonies)

Blood agar not hemolyzed

Milk (with iron strip) Slow fermentation, with a stream of fine gas bubbles, coagulation at 18 to 20 hours, with the coagulum torn and forced to the surface No digestion or blackening even upon prolonged incubation

Indole is not formed

Acid and gas from glucose, maltose, galactose, and mannitol Lactose more slowly fermented Sucrose, salicin, dulcitol, and inositol not fermented

Nitrates are not reduced to nitrites

Coagulated albumin not liquefied

Blood serum not liquefied

Brain medium (Hibler) Not blackened nor digested, even in the presence of an iron strip

Lead acetate agar or peptone iron agar Not blackened

Nonpathogenic for white mice, guinea pigs, and rabbits

Optimum temperature Not determined, grows well at both 37 C and at room temperature

Outstanding character The extremely gummatous character of the submerged colonies

Strictly anaerobic

Source Isolated once from gaseous gangrene and twice from normal human feces (adult and infant)

Habitat Not determined other than from these sources

#### SUMMARY

A detailed description is presented of three anaerobic species that are believed not to have been previously isolated or identified To these there have been assigned names appropriate to their morphologic and physiologic characteristics, namely, *Clostridium nauseum*, *Clostridium microsporum*, and *Clostridium gummosum*

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# THE SUSCEPTIBILITY OF PENICILLINASE-PRODUCING BACTERIA TO PENICILLIN

## I FACTORS INFLUENCING SUSCEPTIBILITY

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Penicillinase, an enzyme that destroys penicillin (Abraham and Chain, 1940), is produced by a wide variety of bacteria (Bondi and Dietz, 1944a). Considerable variation is encountered among these organisms as to their susceptibility to penicillin (Bondi and Dietz, 1944b). Some strains that produce this enzyme are susceptible to penicillin (Bondi and Dietz, 1946). It appeared worth while to study a wide variety of such organisms to determine further the factors that influence their susceptibility.

### EXPERIMENTAL PROCEDURES

The susceptibility of the various organisms studied was determined by a tube dilution technique. Series of tubes of 10 per cent tryptone broth carrying two-fold concentrations of penicillin were inoculated with 18- to 24-hour broth cultures of organisms under investigation. Crystalline potassium penicillin G was used throughout the work. Since inoculum size has been shown to influence the susceptibility of these organisms (Kirby, 1945; Luria, 1946), at least three different inocula were tested. The tubes were incubated at 37 C, and growth was read in 24 and 48 hours. All cultures studied were strains that had been maintained in a laboratory stock culture collection for several years on veal infusion agar slants.

### EXPERIMENTAL RESULTS

Table 1 shows the results of susceptibility tests on two gram-positive and two gram-negative penicillinase-producing organisms. For comparison, the results of similar tests on one gram-positive and one gram-negative non-penicillinase-producing organisms are shown. A sharp difference in susceptibility is encountered between the two groups of organisms that produce the enzyme. Small inocula of the gram-positive strains are quite susceptible. In fact, individual cells of these organisms have the same order of susceptibility as do those of *Staphylococcus H*, which does not produce penicillinase. Larger inocula are less sensitive because of the more rapid multiplication of the cells resulting in greater production of enzyme and the subsequent rapid destruction of penicillin (Kirby, 1945). Similar results are obtained when these large inocula consist of washed

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TABLE 1  
The susceptibility of penicillinase-producing bacteria

ORGANISM	INOCULUM 0.1 ML	INHIBITION OF GROWTH BY PENICILLIN—UNITS/ML											
		None	0.04	0.08	0.16	0.32	0.64	1.25	2.50	5.0	10.0	20	40
<i>Staphylococcus</i> 163	10 <sup>-1</sup>	4*	4	4	4	4	4	4	4	2	—	—	—
	10 <sup>-3</sup>	4	4	4	4	—	—	—	—	—	—	—	—
	10 <sup>-5</sup>	4	4	4	—	—	—	—	—	—	—	—	—
<i>B. anthracis</i> "E"	10 <sup>-1</sup>	4	4	4	4	—	—	—	—	—	—	—	—
	10 <sup>-3</sup>	4	2	—	—	—	—	—	—	—	—	—	—
	10 <sup>-5</sup>	4	—	—	—	—	—	—	—	—	—	—	—
<i>Staphylococcus</i> H†	10 <sup>-1</sup>	4	4	2	—	—	—	—	—	—	—	—	—
	10 <sup>-3</sup>	4	2	—	—	—	—	—	—	—	—	—	—
	10 <sup>-5</sup>	4	2	—	—	—	—	—	—	—	—	—	—
<i>Shigella dysenteriae</i>	10 <sup>-1</sup>	4	4	4	4	4	4	4	4	4	4	2	—
	10 <sup>-3</sup>	4	4	4	4	4	4	4	4	4	4	—	—
	10 <sup>-5</sup>	4	4	4	4	4	4	4	4	4	—	—	—
<i>E. coli</i>	10 <sup>-1</sup>	4	4	4	4	4	4	4	4	4	4	4	2
	10 <sup>-3</sup>	4	4	4	4	4	4	4	4	4	4	4	—
	10 <sup>-5</sup>	4	4	4	4	4	4	4	4	4	4	—	—
<i>E. typhosa</i> †	10 <sup>-1</sup>	4	4	4	4	4	4	4	2	—	—	—	—
	10 <sup>-3</sup>	4	4	4	4	4	4	2	—	—	—	—	—
	10 <sup>-5</sup>	4	4	4	4	4	4	2	—	—	—	—	—

\* 4 = no inhibition of growth, 2 = partial inhibition of growth, — = complete inhibition of growth

† Do not produce penicillinase

TABLE 2  
The susceptibility of penicillinase-producing gram-positive bacteria

ORGANISM	INHIBITING CONCENTRATION OF PENICILLIN UNITS/ML			
	0.1 ml inoculum dilutions			
	Undil	1 × 10 <sup>-2</sup>	1 × 10 <sup>-4</sup>	1 × 10 <sup>-6</sup>
<i>Staphylococcus</i> 7729	>10.0	5.0	1.28	0.16
<i>Staphylococcus</i> 161	>10.0	5.0	0.40	0.16
<i>B. anthracis</i> E	2.56	0.32	0.02	<0.02
<i>B. anthracis</i> Y	1.28	0.08	0.02	—
<i>B. cereus</i>	>10.0	>10.0	5.0	1.28
<i>Bacillus</i> sp	>10.0	>10.0	2.5	—
<i>Bacillus</i> sp	>10.0	0.64	0.16	—
<i>B. megatherium</i>	1.28	0.02	<0.02	<0.02

cells, indicating, as shown by Luria (1946), that the greater destruction of penicillin is not due to carry-over of penicillinase in the inocula

The same relationship does not hold true for the gram-negative bacilli that produce penicillinase. Inoculum size does not influence their susceptibility to the extent that it does the gram-positive organisms. Small inocula are not susceptible. Although production of this enzyme probably contributes to the resistance of these organisms, it is not the primary factor. Some other mechanism common to all gram-negative organisms, with the exception of *Neisseria gonorrhoeae* and *Neisseria intracellularis*, is primarily responsible for their resistance.

Table 2 shows the susceptibility of a number of additional gram-positive bacteria. The effect of inoculum is evident. A wide variation in susceptibility is apparent. Some cultures, such as *Bacillus megatherium* and *Bacillus anthracis*, are highly susceptible when smaller inocula are tested. Others, such as *Bacillus cereus*, are resistant.

TABLE 3

*The relationship of susceptibility to quantitative penicillinase production*

ORGANISM	INOCULUM 0.1 ML	PENICILLIN—UNITS/ML		
		Inhibiting concentration	Destruction/ml*	
			24 hr culture	96-hr culture
<i>Staphylococcus</i> 161	10 <sup>-2</sup>	5 0	15 36	22 0
	10 <sup>-4</sup>	0 40		
	10 <sup>-6</sup>	0 16		
<i>B. megatherium</i>	10 <sup>-2</sup>	0 02	7 67	76 8
	10 <sup>-4</sup>	<0 02		
	10 <sup>-6</sup>	<0 02		
<i>B. cereus</i>	10 <sup>-2</sup>	>10 0	7 68	29 4
	10 <sup>-4</sup>	5 0		
	10 <sup>-6</sup>	1 28		

\* Incubation for 1 hour at 37 C

It appeared likely that susceptibility was related directly to the ability of an organism to produce penicillinase quantitatively. To obtain evidence of this, 24- and 96-hour broth cultures of three organisms with varying degrees of susceptibility were assayed for penicillinase by a method previously described (Bondi and Dietz, 1944a). These results are shown in table 3. Enzyme production is expressed in terms of the amount of penicillin destroyed by 1.0 ml of the culture. The susceptibilities of these cultures are likewise shown for comparison. It is apparent that there is not a direct relation between the amount of penicillinase produced by an organism and its susceptibility to penicillin. *Bacillus megatherium*, which is the best producer of the enzyme, is the most susceptible of the three cultures tested. The resistance of these organisms does not appear to be directly related to their ability potentially to produce large amounts of the enzyme.

Organisms vary considerably in the speed with which they produce penicillinase. As shown in table 3, the yield of penicillinase from a culture of *B. megatherium* was increased tenfold by incubation for 4 days. It seemed likely that the speed with which an organism produced this enzyme and the subsequent rapid destruction of penicillin had a direct bearing on its susceptibility to penicillin. Evidence for this is shown in table 4. At intervals shortly after a series of tubes containing twofold concentrations of penicillin had been inoculated with varying inocula of these organisms for susceptibility testing, assays were made from certain of the tubes for residual penicillin. The results of the assays from the tubes containing 5.0 units per ml 1, 2, and 4 hours after inoculation are shown. It is evident that there is a correlation between resistance and the rapidity of penicillin destruction. *B. megatherium*, the most susceptible of the organisms

TABLE 4

*The relationship of susceptibility to speed of production of penicillinase*

ORGANISM	INOCULUM 0.1 ML	INHIBITING CONCENTRATION, UNITS/ML	PENICILLIN—5.0 UNITS/ML BROTH		
			Units/ml left		
			1 hr	2 hr	4 hr
<i>Staphylococcus</i> 161	Undil	>10.0	2.9	None	None
	10 <sup>-2</sup>	5.0	4.9	4.6	4.15
	10 <sup>-4</sup>	40	5.2	5.0	5.10
<i>B. megatherium</i>	Undil	1.28	4.7	4.2	3.0
	10 <sup>-2</sup>	.02	5.0	4.9	4.8
	10 <sup>-4</sup>	<.02	5.3	5.1	4.9
<i>B. cereus</i>	Undil	>10.0	1.75	None	None
	10 <sup>-2</sup>	>10.0	4.80	4.9	None
	10 <sup>-4</sup>	5.0	5.30	5.2	4.7

studied in this fashion, destroyed relatively small amounts of penicillin during the short intervals tested. *B. cereus*, however, which is resistant, rapidly destroyed the 5.0 units present.

#### DISCUSSION

Undoubtedly, penicillinase production contributes to the resistance of a gram-positive organism to penicillin. The degree of its resistance depends upon the ability of the organism to produce the enzyme rapidly rather than upon its ability potentially to produce large amounts of it. If a sufficient amount is produced by an organism to destroy the antibiotic present soon after contact, growth rather than inhibition of the organism takes place.

Individual cells of many gram-positive penicillinase-producing organisms are as susceptible to penicillin as are those of organisms that do not produce the enzyme. The therapeutic effectiveness of penicillin in the treatment of an infection due to such an organism, apart from dosage, will depend not only upon the



number of organisms present but upon the speed with which they produce the enzyme. Anthrax, caused by a gram-positive penicillinase-producing organism, has responded well to penicillin therapy (Murphy, LaBocchetta, and Lockwood, 1944). *In vitro* tests have shown that *B. anthracis* is a very slow producer of penicillinase and consequently is susceptible, particularly when small inocula are tested.

Penicillinase production likewise contributes to the resistance of the gram-negative penicillinase-producing organisms. However, some other basic factor common to most gram-negative organisms is primarily responsible for their resistance. The nature of this resistance is unknown.

#### SUMMARY

The susceptibility to penicillin of a wide variety of penicillinase-producing organisms is reported.

Considerable variation in the susceptibility of the gram-positive organisms is observed, individual cells of some of these cultures are highly sensitive. The degree of resistance of these organisms is related to the speed with which they produce the enzyme rather than to the amount that they produce.

Although production of this enzyme may increase the resistance of gram-negative organisms, it is not the primary factor in their resistance to penicillin.

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# THE SUSCEPTIBILITY OF PENICILLINASE-PRODUCING BACTERIA TO PENICILLIN

## II THE EFFECT OF SODIUM AZIDE

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In a previous publication Bondi and Dietz (1947) reported that various factors could influence the susceptibility of penicillinase-producing bacteria to penicillin. The authors concluded that individual cells of certain organisms that produce this enzyme may be just as susceptible to penicillin as those of organisms that do not produce penicillinase. In a continuation of this study, the effect of sodium azide on the susceptibility of penicillinase-producing organisms has been studied to determine further the mechanisms that influence their susceptibility to this antibiotic.

Woodruff and Foster (1945) studied the effect of various agents on penicillinase activity and found ferrous chloride, iodoacetic acid, and sodium azide to have an inhibitory effect. Two groups of workers, Benedict, Schmidt, and Coghill (1945) and Henry and Housewright (1947), were unable to show an effect by sodium azide on this enzyme. Tieffers (1946), in studying the potentiation of penicillin by various agents, concluded that sodium azide, iodoacetic acid, and many other compounds could enhance the action of penicillin without involving the inhibition of penicillinase.

The sodium azide used in this study was prepared from a 1 per cent stock solution in sterile distilled water. This solution was allowed to remain at room temperature overnight and added aseptically to 1 per cent tryptone broth to give the desired concentration. Stock solutions of crystalline potassium penicillin G in sterile saline, 2,000 Oxford units per ml, were kept frozen until used. Fresh penicillin solutions, in the concentrations desired, were prepared from the stock solutions regularly.

The cultures of staphylococci studied were taken either from a stock culture collection or isolated from routine hospital specimens. *Staphylococcus* X-3 "resistant" was obtained by isolation of a resistant variant from the parent culture by passage through broth containing increasing concentrations of penicillin. The latter organism did not gain the ability to produce penicillinase.

Preliminary experiments were run to determine the highest concentration of sodium azide that would not appreciably affect the growth of the organism tested. This was accomplished by adding different concentrations of the chemical to 1 per cent tryptone broth and inoculating sets of such broths with cultures of staphylococci. From the results, summarized in table 1, 0.02 per cent sodium

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azide was chosen as the highest concentration that could safely be used without killing the organisms, although some inhibition of growth by this concentration was observed

In the first series of experiments, the penicillin susceptibilities of 6 strains of staphylococci (3 penicillinase and 3 non-penicillinase-producers) in the presence and absence of sodium azide were determined, as summarized in table 2. This was done by adding different concentrations of penicillin to tubes of 1 per cent tryptone broth with and without sodium azide in final concentrations of 0.02 per cent, 0.01 per cent, and 0.005 per cent. All tubes were inoculated with a drop of

TABLE 1  
*Inhibition of growth of staphylococci by sodium azide*

STAPHYLOCOCCUS	DILUTION OF INOCULUM	GROWTH INHIBITION BY VARYING CONCENTRATIONS OF SODIUM AZIDE					
		None	0.005%	0.01%	0.02%	0.04%	0.08%
H	Undiluted 1/100	4*	4	3	3	2	—
		4	4	3	3	2	—
X-3 "Susceptible"	Undiluted 1/100	4	4	4	4	3	—
		4	3	3	3	—	—
X 3 "Resistant"	Undiluted 1/100	4	3	3	3	—	—
		4	3	2	1	—	—
No 446†	Undiluted 1/100	4	3	3	3	3	—
		4	3	3	2	1	—
No 7815†	Undiluted 1/100	4	4	4	4	3	—
		4	4	4	4	2	—
No 1752†	Undiluted 1/100	4	3	3	3	2	—
		4	3	3	3	1	—

\* 4 = no inhibition of growth, 3, 2, and 1 = varying degrees of inhibition of growth,  
— = complete inhibition of growth

† Produce penicillinase

a 24-hour broth culture of the organism being studied or a 1/100 dilution of that culture. An effect was observed with those staphylococci capable of producing penicillinase that was not seen with those that did not. The former were rendered approximately 15 times more susceptible to penicillin in the presence of 0.02 per cent sodium azide when large inocula were used. The results with staphylococci, incapable of producing this enzyme, are strikingly different. The latter organisms, if affected at all, became at most four times as susceptible as the controls. The resistant variant of *Staphylococcus* X-3 behaved as the rest of the non-penicillinase-producers, despite its resistance to penicillin. The potentiation of penicillin on penicillinase-producing organisms is reduced when the inoculum is small and the concentration of sodium azide is decreased.

From the foregoing experiment, it is concluded that sodium azide had an effect on penicillinase-producing staphylococci over and above that which may be exerted against non-penicillinase-producers. This seemed to indicate some interference with the enzyme by this chemical.

TABLE 2

*Effect of sodium azide on penicillin sensitivity of staphylococci*

PENICILLINASE PRODUCERS				NON PENICILLINASE PRODUCERS			
<i>Staphylococci</i>	Conc of sodium azide %	Dilution of inoculum	Inhibiting concentration of penicillin u/ml	<i>Staphylococcus</i>	Conc of sodium azide %	Dilution of inoculum	Inhibiting concentration of penicillin u/ml
No 1752	0.02	Undil $10^{-2}$	6.4 0.1	H	0.02	Undil $10^{-2}$	0.025 0.013
	0.01	Undil $10^{-2}$	102.0 0.4		0.01	Undil $10^{-2}$	0.025 0.025
	None	Undil $10^{-2}$	102.0 0.8		None	Undil $10^{-2}$	0.05 0.025
No 7990	0.02	Undil $10^{-2}$	6.4 0.4	X-3 "Susceptible"	0.02	Undil $10^{-2}$	0.1 X
	0.01	Undil $10^{-2}$	25.6 0.4		0.01	Undil $10^{-2}$	0.10 0.05
	None	Undil $10^{-2}$	>102.0 0.8		None	Undil $10^{-2}$	0.1 0.1
No 7768	0.02	Undil $10^{-2}$	6.4 0.4	X-3 "Resistant"	0.02	Undil $10^{-2}$	12.8 X
	0.01	Undil $10^{-2}$	12.8 0.4		0.01	Undil $10^{-2}$	51.2 25.6
	None	Undil $10^{-2}$	102.0 0.4		None	Undil $10^{-2}$	51.2 12.8

X = no growth in sodium azide control

To study the mechanism of action of sodium azide, experiments were run to correlate the rate of growth of penicillinase-producing organisms with the rate of destruction of penicillin. To flasks containing 50 ml of 1 per cent tryptone broth were added penicillin to give a final concentration of 0.75 units per ml, and sodium azide to give the concentrations required. All flasks were then inoculated with 0.5 ml of a 24-hour broth culture. Samples were removed at intervals of 0, 1, 2, 4, and 8 hours and heated at 60°C for 30 minutes to stop growth of the organism and to inactivate the penicillinase that might be present. Penicillin assays were

run by the Oxford cup technique, and the percentage of destruction was calculated. Growth was simultaneously determined by measuring optical densities

TABLE 3

*Relationship of growth to penicillin destruction in the presence of sodium azide*

CONCENTRATION of NaN <sub>3</sub>	CONCENTRATION OF PENICILLIN u/ml	TIME INTERVAL									
		0 hr		1 hr		2 hr		4 hr		8 hr	
		Pen des %	Opt den	Pen des %	Opt den	Pen des %	Opt den	Pen des %	Opt den	Pen des %	Opt den
Staphylococcus 7768											
None	None		0		6		10		52		129
None	0.75	0	4	49	4	100	5	100	9	100	111
0.02	0.75	0	2	0	1	75	1	99	4	100	5
0.01	0.75	0	2	0	5	100	6	100	5	100	54
0.005	0.75	0	2	49	0	100	0	100	9	100	75
Staphylococcus 1752											
None	None		0		7		14		64		143
None	0.75	0	5	47	5	94	5	100	11	100	105
0.02	0.75	0	2	36	1	67	1	100	0	100	3
0.01	0.75	0	3	36	3	76	2	100	3	100	17
0.005	0.75	0	0	47	0	86	0	100	0	100	52

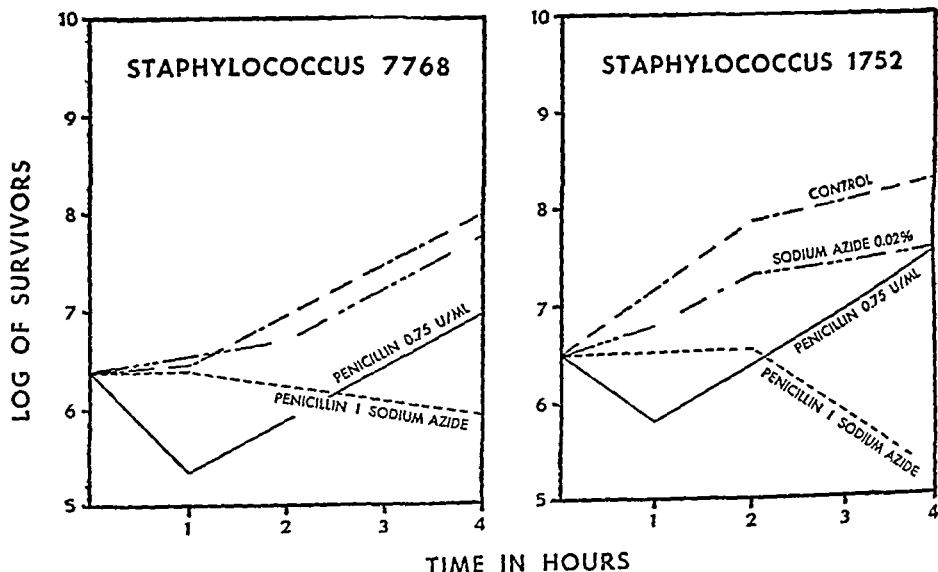


FIG 1 BACTERICIDAL EFFECTS OF PENICILLIN AND SODIUM AZIDE COMBINED

on a Klett-Summerson photometer. Table 3 summarizes the results obtained with two strains of staphylococci. Similar results were obtained with two other

strains of staphylococci. It was noted that the penicillin in flasks containing no sodium azide, or small concentrations of the chemical, was destroyed more rapidly than the penicillin in flasks containing higher concentrations of sodium azide. Growth occurred after penicillin destruction, and the rate of growth varied with the speed with which penicillin was destroyed. It was also observed that penicillin destruction occurred in the absence of detectable growth. The concentration of penicillin used, 0.75 units per ml, was chosen because it afforded an experiment that could be concluded in one day. The results of a similar experiment, using higher concentrations of the antibiotic, were essentially the same except that the period of time for penicillin destruction and hence for growth to begin is prolonged.

Since turbidimetric readings give evidence only of bacteriostatic action, bacterial counts were made to study the bactericidal activity of penicillin-azide combinations. To flasks of 1 per cent tryptone broth in volumes of 50 ml containing either penicillin, sodium azide, or both, were added 0.5 ml of an undiluted

TABLE 4  
*Effect of  $\text{NaN}_3$  on sterile penicillinase of Staphylococcus 7990*

CONCENTRATION OF PENICILLIN u/ml	CONCENTRATION OF $\text{NaN}_3$ %	PENICILLIN DESTROYED, PERCENTAGE				
		$\frac{1}{2}$ hr	1 hr	2 hr	3 hr	4 hr
0.75	0	36	61	73	93	100
0.75	0.02	20	58	86	95	100
0.75	0.01	20	63	76	86	100
0.75	0.005	20	60	67	86	100

24-hour broth culture. Bacterial counts were made at the end of 0, 1, 2, 4, and 6 hours on extract agar. These counts, shown in figure 1, demonstrate that sodium azide alone did not appreciably affect growth. Penicillin alone surprisingly caused a drop in count in the first hour of the experiment, but later the number of viable organisms followed a pattern resembling that of the control. A combination of the two agents was followed by an appreciable drop in the total count that persisted for 24 hours.

From the results of these experiments, it is apparent that the destruction of penicillin is delayed by the presence of sodium azide. To determine whether this delay is due to direct interference with penicillin activity or a slowing up of the production of the enzyme the following experiments were run. Sterile penicillinase was prepared by the method of Harper (1943) from *Staphylococcus* 7990 and stored in sterile tubes. One-half ml of this material was added to flasks of 1 per cent tryptone broth, containing 0.75 units per ml of penicillin and varying concentrations of sodium azide. Penicillin assays were run at  $\frac{1}{2}$ , 1, 2, 3, and 4 hours, and the penicillin destroyed was calculated.

From table 4 it is evident that the rate of destruction was the same in flasks containing azide as in the control flasks. Similar results were obtained using penicillinase prepared from *Staphylococcus* 1752. These results indicate that

sodium azide does not interfere with the rate of destruction of penicillin by pre-formed enzyme. Therefore the action is probably due to a slowing down or interference with the production of penicillinase.

#### DISCUSSION

There may be some synergistic or additive effect of combinations of sodium azide and penicillin on all staphylococci studied. Over and above this effect, sodium azide renders penicillinase-producing organisms more susceptible to penicillin. That the action of sodium azide is related to penicillinase is indicated by its greater action on penicillinase producers than on the non-penicillinase-producers that are more susceptible to penicillin and equally sensitive to sodium azide. It is significant to note that *Staphylococcus* X-3, which *in vitro* was made resistant to penicillin but which did not gain the ability to produce penicillinase, was not rendered sensitive in the presence of sodium azide.

Two possible mechanisms for the action of sodium azide seemed apparent at the beginning of this study. Either the action of penicillinase was inhibited or the production of the enzyme by a growing culture was suppressed. The results obtained indicate that penicillin destruction by sterile preparations of the enzyme proceeds at the same rate regardless of the presence of sodium azide in the concentration used. This indicates that the potentiating action of this agent is probably due to a suppression of the production of penicillinase by growing cultures rather than to an inhibition of the enzyme. Although sodium azide is much too toxic to be safely used *in vivo*, it is possible that a compound exerting comparable activity but lower toxicity could be used effectively in conjunction with penicillin to treat infections due to penicillinase-producing staphylococci.

#### SUMMARY

Sodium azide renders penicillinase-producing staphylococci more susceptible to penicillin. The action of this agent is not due to an interference in the activity of penicillinase as indicated by its failure to act in this fashion on sterile preparations of the enzyme. Penicillinase production by a growing culture of *Staphylococcus* is suppressed by sodium azide.

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# THE EFFECTS OF AN EXTRACT OF BLOOD CELLS UPON THE CULTIVATION OF BACTERIUM TULARENSE IN LIQUID MEDIA

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Until satisfactory synthetic media become available, a clear liquid medium of defined composition that will support sustained growth from small inocula and provide large yields of cells would fill many needs. Further study and exploitation of the properties of an aqueous extract of blood cells have resulted in the incorporation of these characteristics into improved liquid media of the type devised by Tamura and Gibby (1943). The constituents of unknown composition are the acid hydrolyzates of the selected proteins and the blood cell extract (BCE). For this study we are not concerned with the composition of BCE but with certain of its properties and practical uses.

*Preparation of blood cell extract* Although satisfactory extracts were prepared from rabbit and horse bloods, most were made from human blood, using cells obtained from the hospital's blood bank. Nine volumes of distilled water were added to each volume of packed, unwashed cells. The laked suspension was heated with constant stirring and boiled for 3 minutes. While a temperature between 95 and 100 C was maintained, concentrated HCl was added to bring the pH to 6.3. After acidification this temperature was maintained for 5 minutes, whereupon, while still hot, the coagulum was removed by filtration through fluted paper. The fluid was refiltered through the same paper until the filtrate was clear. The volume was recorded as filtrate A, of which each 10 ml represented 1 ml of original cells. This filtrate was concentrated in a vacuum still to one-fiftieth or less of its volume. The concentrate was restored to pH 7.0, heated to the boiling point, and filtered through paper until clear. The filter was well drained, and the volume of the clear filtrate was recorded as filtrate B. This liquid concentrate was used for current cultural needs. The ratio of the volumes of filtrates B to A was used to calculate the volume of filtrate B that was required per liter of medium to give a concentration of BCE of any desired percentage in terms of the original packed blood cells. For example, if filtrate A was concentrated to one-fiftieth of its volume, each 1 ml of filtrate B was equivalent to 5 ml of blood cells. Hence for a medium to contain 5 per cent BCE in terms of original cells, it required 10 ml of concentrate per liter.

Storage of unsterilized BCE concentrate at 5 C was unsatisfactory except for short periods. Preservation without loss of activity was accomplished either by rapid freezing and storage in a dry ice chest or by dehydration from the rapidly frozen state. The lyophilized material offered the advantages of safe storage of the ground powder in stoppered bottles and of gravimetric additions to media.

*Basal media* Two basal media were used to study the metabolic properties of BCE Their formulas were

Basal medium A

	per L
Gelatin hydrolyzate to make N content	3 0 g
Cystine	0 1
Glycerol	5 0
NaCl (total)	15 0
Na <sub>2</sub> HPO <sub>4</sub> 12 H <sub>2</sub> O	0 625
KH <sub>2</sub> PO <sub>4</sub>	0 25
MgSO <sub>4</sub>	0 025

Basal medium B

	per L
Gelatin hydrolyzate to make N content	3 0 g
Cystine	0 1
Glycerol	5 0
NaCl (total)	15 0

Substitutions were made in basal medium B as follows hydrolyzates of crude casein, vitamin test casein, or of a partly defatted soybean protein for gelatin hydrolyzate, glucose for glycerol The hydrolyzates were prepared, with slight modifications, according to the method of Williams *et al* (1941) Since neutralized acid hydrolyzates varied in chloride content, it was necessary to determine chlorides (as NaCl) for each lot The difference between each determination and the totals recommended in the media above must be made up by additional NaCl Most strains tested here grew better and faster with glycerol than with glucose, but some grew just as well with glucose, and an occasional strain seemed to prefer it All media were adjusted to an initial pH of 6.9 unless otherwise stated

*Culture tubes* Provision for a high ratio of surface to volume of media, and for frequent determination of growth by means of the turbidity of cultures, was made by blowing bulbs of  $40 \pm 1$  mm diameter from the closed ends of 18-mm pyrex tubes, and by sealing 16-cm side arm tubes, made from selected pyrex tubing of  $10.5 \pm 0.12$  mm outside diameter, into the large tubes just above the level of the bulbs The distal ends of the side arms were sealed These side arm tubes fitted vertically into the holder of a photoelectric turbidity comparator Cultures could be tipped into the side arms at will for turbidity measurements without risk of contamination

*Inocula for cultures* Unless otherwise stated all inocula contained about 40 millions of thrice-washed cells in 0.1-ml volumes They were prepared from 24-hour cultures in gelatin hydrolyzate medium All cultures were incubated at 37 C Most work was performed with strains Memp and Schu, occasionally others were used

*Method for quantitative determination of growth* A fairly accurate approximation of the number of bacteria per unit volume was obtained with the photoelectric comparator described by Krebs and associates (1942) Measurements of turbidity were obtained in terms of microamperes (M A) Many virulence titrations in mice indicated that suspensions in 10.5-mm tubes, adjusted to 24.0 M A, contained 2.5 to 3 billions of bacteria per ml Calibration of the instru-

ment with dilutions of bacterial suspensions, and with dilutions of standard silicate suspensions, showed that M A readings were almost directly proportional to turbidity throughout its range. Table 1 indicates the approximate numbers of bacteria in suspensions with various M A readings.

*Some physical and chemical properties of BCE* The color of dried extract varies from light yellow-brown to light reddish-brown. It is readily soluble in water. Although its composition may vary from lot to lot, a large representative sample gave the following test results. The biuret and xanthoproteic tests were faintly positive. The Molisch and Bial tests were strongly positive. Benedict's test was negative. Sulfur and phosphorus were present, and nitrogen formed 5.4 per cent of the dry weight. It was irreversibly reduced, with bleaching of

TABLE 1

*Approximate equivalents of turbidity in microamperes measured in 10 5-mm tubes and numbers of Bacterium tularense*

Microamperes	Bact./ml in millions
2.5	150
4	330
6	600
8	909
10	1,210
12	1,365
15	1,670
20	2,120
24	2,500
30	3,030
36	3,485
42	4,015
50	4,550

color, by sodium hydrosulfite. In 8 per cent aqueous solution there was no light absorption in the visible spectrum.

*Physiological properties of BCE, effect upon growth* The addition of BCE to a basal medium that will not support growth of *Bacterium tularense* with large washed inocula gives it a capacity for sustained multiplication from small washed inocula. The growth-promoting effects of ascending concentrations of a typical sample in basal medium A are shown in table 2. The inoculum for each culture contained about 50 million organisms. Media made from hydrochloric acid hydrolyzates of gelatin supported sustained multiplication from washed inocula that contained from 5 to 10 organisms, to 5 ml of medium, on repeated testing. Identical media made with sulfuric acid hydrolyzates or from hydrolyzates of other proteins were not capable of initiating growth from such small inocula.

*Stabilizing effect of BCE on the pH of culture media* One of the difficulties early encountered in the cultivation of this organism in liquid media was the extraordinary and unpredictable fluctuations of the pH of cultures. In some cultures

end points of pH 4.8 were reached after 24 hours of incubation, invariably with very scanty growth. In others, usually those with good growth, end points of pH 8.3 occurred, which caused cessation of multiplication. These difficulties with still cultures were magnified in degree and accelerated in appearance by either shaking or aeration. The stabilizing effect of BCE upon the pH after 72 hours of incubation is also shown in table 2, in which turbidity and pH determinations are tabulated against increasing increments of BCE in otherwise identical cultures. Since these pH determinations were made with bromthymol blue, it is probable that the two upper readings were higher than the values actually reached.

TABLE 2

*The effect of increasing concentrations of blood cell extract on growth and on pH values in basal medium A after incubation for 72 hours*

BCE PER CENT	M A	pH
0	5	6.0 or less
1	23	6.0 or less
2	32	6.0
3	36	6.2
4	39	6.4
5	41	6.6
6	40	6.7
7	40	6.7
8	43	6.7
9	46	6.8
10	47	6.8
12.5	46	7.0
15	49	7.1
17.5	52	7.1
20	49	7.1

Many attempts were made to find suitable buffers to stabilize the pH of growing cultures. Phosphate buffers that maintained reactions at or near neutrality in concentration of 0.03 M, but not in lesser concentrations, inhibited growth in concentrations above 0.02 M. Carbonate and acetate buffers gave similar results, growth was approximately inversely proportional to the concentrations of the buffers. Sodium glycinate did not inhibit growth, nor did it stabilize the pH. Variations in concentrations of hydrolyzates of gelatin or of casein were also ineffective. No stabilizing buffer system was found that did not adversely affect growth. It was even shown that the amounts of phosphates present in basal medium A were inhibitory, and that increased yields of cells were obtained after they and the magnesium salt were omitted.

Since BCE had revealed a stabilizing effect upon the pH levels of growing cultures, its capacity was tested further by adding it in 10 per cent concentration to basal medium B and making a duplicate series of tubes with initial pH values ranging from 5.0 to 7.8 in steps of 0.4. Turbidity measurements and pH deter-

minations were made daily for 3 days. These measurements are shown in table 3 and figure 1. All cultures within the initial pH range of 5.8 to 7.8 developed

TABLE 3

*Growth and pH changes of cultures with different initial pH values during 3 days of incubation in basal medium B containing 10 per cent of BCE*

INITIAL pH	pH AT 24 HR	M A AT 24 HR	pH AT 48 HR	M A AT 48 HR	pH AT 72 HR	M A AT 72 HR
5.0	4.8	1	4.8	2	4.8	2
5.0	4.8	1	4.8	2	4.8	2
5.4	5.3	1	5.3	2	5.4	2
5.4	5.3	1	5.3	2	5.4	2
5.8	5.9	10	6.9	27	7.0	37
5.8	5.9	10	6.9	28	7.0	37
6.2	6.2	10	7.0	26	6.8	36
6.2	6.4	10	7.0	27	7.0	38
6.6	6.9	8	6.9	27	7.0	38
6.6	6.9	8	6.9	27	7.0	38
7.0	7.0	8	6.9	26	6.8	40
7.0	7.0	8	6.9	26	6.8	39
7.4	7.4	5	6.9	18	6.8	33
7.4	7.4	5	6.9	19	6.8	37
7.8	7.8	3	6.9	10	6.9	25
7.8	7.8	3	6.9	10	6.9	24

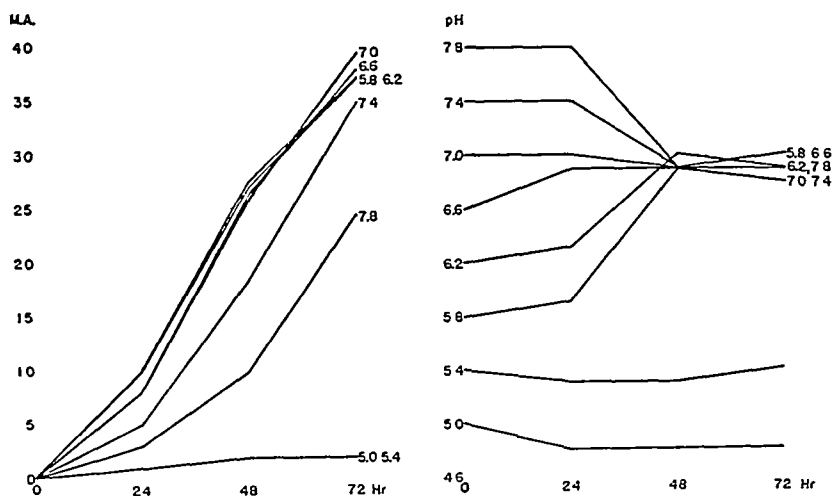


FIG 1 THE GRAPH AT THE LEFT ILLUSTRATES THE RATE OF GROWTH AND THE TERMINAL DENSITIES IN TERMS OF MICROAMPERES OF THE DUPLICATE CULTURES WHOSE INITIAL pH VALUES, AND DAILY pH VALUES FOR 3 DAYS OF INCUBATION, ARE SHOWN AT THE RIGHT

values that steadily approached neutrality. Those within the 5.8 to 7.0 pH range multiplied rapidly and steadily. Even those with initial pH values of 7.4 gave good yields after 72 hours although they grew more slowly. The 7.8 cul-

tures grew still more slowly and failed to give satisfactory yields. Cultures with initial pH values of 5.4 or less showed little or no growth. Further studies confirmed that BCE alone supplied substances that not only markedly stimulated growth but also stabilized the pH of unbuffered gelatin or casein hydrolyzate cultures.

*The mechanism of pH stabilization of cultures by BCE* The addition of BCE to uninoculated media does not alter the pH. The remote likelihood that it could serve as a buffer was shown to be untenable. Serial tubes of basal medium B containing ascending quantities of BCE from 1 to 5 per cent were adjusted to a pH of 6.9, were inoculated with washed cells and incubated for 72 hours, whereupon turbidity, pH values, and titratable acidity values were determined. Cultures containing 1 per cent of BCE showed the least growth, the lowest pH values, and the highest titratable acidities. With ascending quantities of BCE the determinations, recorded in table 4, showed progressively increased growth and pH values, and decreased titratable acidity values. Cultures that contained 5

TABLE 4

*The effect of ascending concentrations of BCE upon growth, pH, and titratable acidity after incubation for 3 days in basal medium B*

(The initial pH of the medium was 6.9)

BCE PER CENT	M. A.	pH	TITRATABLE* ACIDITY
1	21	5.1	2.00
2	31	5.5	1.50
3	34	5.8	0.75
4	39	6.1	0.50
5	40	6.3	0.25

\* Ml of 0.01 N NaOH required to titrate to pH 6.9

per cent BCE showed a fall in pH from 6.9 to 6.3 in 72 hours, and 0.25 ml of 0.01 N NaOH were required to restore the pH to 6.9. Cultures that contained 1 per cent of BCE showed a fall in pH from 6.9 to 5.1 during the same period, and required 2.0 ml of 0.01 N NaOH to restore the pH to 6.9. If BCE had exerted a direct buffering action the cultures containing 5 per cent should have produced as much, if not more, titratable acid as did the cultures containing 1 per cent BCE, whereas the latter actually produced eight times as much. Hence the bufferlike action of BCE on growing cultures is a result of its effects on bacterial metabolism, apparently dependent upon some component of the extract that accelerates amino acid utilization with resultant formation of base, this process keeping pace with and offsetting the effect of acid production from carbohydrate oxidation, including glycerol, if an appropriate concentration of BCE is present. Repeated tests failed to detect a trace of volatile base during the growth of still or aerated cultures that became progressively more alkaline.

*Dialyzability and heat stability of the metabolically active components of BCE* Dialysis of BCE in a cellophane sack against distilled water in a small vessel

disclosed that both the growth-promoting and the pH-stabilizing components disappeared from the sack and appeared in the dialyzate within 24 hours

A sample of concentrated liquid BCE was adjusted to pH 6.9, brought quickly to boiling, and then rapidly cooled in running water. The precipitate was sedimented by centrifugation. A portion of the supernatant was removed for assay, and the remainder was autoclaved for 10 minutes at 10 pounds pressure. The precipitate was sedimented from the autoclaved portion, and the supernatant was removed for assay. Each supernatant was added in increasing amounts to separate series of tubes containing basal medium A, all tubes were adjusted to pH 6.9, and inoculated together with a third series containing identical quantities of unheated BCE. The 72-hour growth yields and the pH values at 24, 48, and 72 hours for each comparable BCE concentration were identical for the boiled and the unheated extracts. The growth yields of the autoclaved series, for each level of BCE used, were about one-third less than those of the corresponding tubes of the other series, and the pH values fell in all tubes to 6.0 or below by 48 hours. Momentary heating to the boiling point did not diminish metabolic activity of the extract, but autoclaving near neutrality destroyed almost all of the pH-stabilizing component.

*Other natural sources of substances with properties similar to those of BCE* A search for substances with growth-promoting and pH-stabilizing properties similar to those of BCE revealed that these functions were present, though to lesser degrees, in extracts prepared from powdered egg yolk, liver cake, yeast, leaf mold, and compost heaps, and marsh and river muds. The extracts were tested in basal media by substitution for BCE.

*Cultural conditions for successful cultivation in liquid media* The demand for a free supply of oxygen must be satisfied. Vessels for still cultures must provide a high ratio of surface area to volume of medium. Suboptimal growth is inevitable from all except large inocula if the medium is dispensed in 5-ml amounts in the usual 18-mm tubes. Growth is still poorer if larger volumes are used. Tubes of 25-mm diameter are satisfactory for 5-ml cultures. Cultures in larger volumes should be grown in Erlenmeyer flasks, using a size that for the desired volume of medium will give a layer of fluid not more than 5 to 8 mm deep.

Aqueous solutions of BCE deteriorated at refrigerator temperatures. Poor or irregular growth resulted from solutions held at 5°C for 6 days. They are more stable in complete media and will hold in that form for 2 weeks at 5°C. The preferred dehydrated powder maintained its activity for many months at room temperatures in tightly stoppered bottles.

#### DISCUSSION

Cultures of *Bacterium tularense* on solid, or in liquid, media produce acids from fermentable carbohydrates, including glycerol, and nonvolatile bases, presumably amines, from amino acids. The reaction of the inoculated medium at any time represents the relative differences in magnitudes and velocities of these processes. Determinations of the pH of cultures may or may not betray evidence of carbohydrate utilization, they are treacherous and unsuitable methods for that pur-

pose The ability of the organism to produce either acidic or alkaline end points in similar media, and indeed first one and then the other during cultivation on a single medium, was also noted by Downs and Bond (1935) and by Francis (1942), who studied the utilization of carbohydrates by means of indicators Growth on highly buffered media is seldom restricted by fluctuations in pH, but in nutritionally adequate but unbuffered liquid media most failures to obtain good yields result from high concentrations of hydrogen or hydroxyl ions In the absence of a buffer system that does not of itself restrict growth it is impossible to say that limitation of growth is not due to exhaustion of an essential substrate or enzyme activator, or possibly to an accumulation of inhibitory end products Since the addition of BCE to liquid cultures that have ceased to grow in the presence of a low pH permits rapid and progressive multiplication for an additional 5 days at 37 C, the importance of waste products is minimized, but, owing to our incomplete knowledge of the composition of the extract, it does not permit discrimination between the effect of an enzyme activator and the exhaustion of an essential substrate that may be present in the extract However, there is convincing evidence that regulation of the reaction of liquid media within the noninhibiting pH range of 6.4 to 7.6 can be effected by appropriate quantities of BCE, and that this regulation depends upon unidentified components that permit the organisms to produce constantly a sufficient quantity of base from amino acids to offset acid production from concurrent oxidation of glucose or glycerol

Exclusive of substances present in basal media, including those made from hydrolyzates of vitamin test casein, BCE apparently supplies all other substrates and growth factors in quantities and proportions that make the complete medium a close approximation to the nutritional needs, for no other simple medium has produced comparable cell yields per unit of time from such small inocula in still cultures

The presence in leaf molds, and in marsh and river muds, of substances with properties similar to those of BCE offers support to the hypothesis suggested by Parker, Steinhaus, and Kohls (1943) that even such an organism as *Bacterium tularense*, generally considered to be fastidious in its nutritional requirements, might, under suitable conditions, persist and multiply in nature outside of animal, insect, and arachnid hosts Subsequent to the epizootic of tularemia among beaver that was reported by Jellison, Kohls, Butler, and Weaver (1942), Parker and his associates demonstrated a prolonged and heavy contamination of river waters in the Northwest that could not reasonably be attributed to successive or continuous wild life sources They showed that marsh mud contained cystine, and that the organism persisted in naturally contaminated, refrigerated mud for 12 weeks The extracts that we prepared from samples of marsh and river muds sent by Dr Parker contained moderate amounts of substances with cultural properties similar to those of BCE Hence, from a nutritional viewpoint there is little reason to doubt that *Bacterium tularense* could maintain itself in muds, and possibly even in waters, in the absence of any living host Since 2 per cent NaCl is not inhibitory to growth, maintenance of the organism is possible in muds covered or bordered by waters of considerable salinity



The media described here, made from acid hydrolyzates of gelatin, soybean protein, casein, or vitamin test casein, and containing 200 to 300 mg per cent of dehydrated BCE, do not neutralize the actions of sulfonamides, PABA, penicillin, or streptomycin. When used with suitable conditions of cultivation they are capable of large bacterial yields. Since all constituents of these media are dialyzable, quantities of cells sufficient for chemical or other analyses are obtainable with relative ease and are entirely free from foreign materials.

The simplest complete medium permits continuous subcultivation at room temperatures with weekly transfer of 0.1 ml of culture to 5 ml of fresh medium with little loss of virulence. Strain Memp was maintained continuously in this medium for more than 2 years.

#### SUMMARY

An aqueous extract of blood cells provided substances that effected a metabolic stabilization of the pH, and supported excellent growth of *Bacterium tularense* from small inocula in simplified, unbuffered, liquid media. The simplest medium contained four ingredients other than the extract and water. This medium is suitable for many strains and many purposes, including the production of large quantities of bacterial cells free from all foreign material.

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# A FURTHER NOTE ON THE ANTIGENIC RELATIONSHIPS OF DONOVANIA GRANULOMATIS (ANDERSON)

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In previous papers (Dunham and Rake, 1948, Rake, 1948) it has been pointed out that a certain percentage (14 per cent) of sera from individuals with chronic superficial nonspecific ulceration (decubitus or varicose) show positive and apparently specific complement fixation with antigen prepared from *Donovania granulomatis*, the etiological agent of granuloma inguinale. It has also been shown (Rake, 1948) that sera from individuals with granuloma inguinale show positive and apparently specific complement fixation with antigen prepared from *Klebsiella pneumoniae* in the same manner as that used for preparation of the antigen from *D. granulomatis*. It was suggested at that time that the results indicated antigenic relationship of the new genus *Donovania* to *Klebsiella*, and further exploration of possible relationships within the tribe *Escherichiae* was promised (Rake, 1948). The apparent false positive complement-fixation reactions in individuals with chronic ulceration were thought to depend on secondary infection of their wounds with organisms showing such antigenic relationships (Rake, 1948). In the present paper results are presented with antigens prepared from *Klebsiella rhinoscleromatis*, *Escherichia coli*, and *Aerobacter aerogenes*, and tested in the complement-fixation test along with antigens from *D. granulomatis* and *K. pneumoniae*.

## MATERIALS AND METHODS

Preparation of the antigens from *D. granulomatis* and *K. pneumoniae* have been described elsewhere, as has the technique of the complement-fixation test employed (Dunham and Rake, 1948, Rake, 1948). The antigens from *K. rhinoscleromatis*, *E. coli*, and *A. aerogenes* were prepared from the sedimented bacterial bodies in the manner described for antigen FB obtained from *K. pneumoniae* as described elsewhere (Rake, 1948). They were used in the test at a dilution of 1:4, at which dilution they were not anticomplementary. For the antigen of *K. rhinoscleromatis*, strain 3 obtained through the courtesy of Dr. E. Hoyt, College of Medical Evangelists, Los Angeles, was used, for the antigen of *E. coli*, strain 56 from our own collection was used, for the antigen of *A. aerogenes*, strain 884 of the American Type Culture Collection was used. In all cases the organisms were grown on beef heart agar prepared as outlined elsewhere (Rake and Oskay, 1948). All antigens were heated in a water bath at 56°C for 1 hour before use. The sera were mostly from cases of granuloma inguinale, other venereal diseases, or chronic superficial ulceration (Dunham and Rake, 1948, Rake, 1948).

The complement-fixation tests were recorded as 4+, 3+, 2+, 1+, or 0 fixation. A serum giving 2+, 3+, or 4+ fixation, with no anticomplementary activity, in a test in which all other controls were satisfactory, was considered positive.

### RESULTS

Table 1 shows the results of complement-fixation tests when antigens prepared from *K rhinoscleromatis*, *K pneumoniae*, *E coli*, and *A aerogenes* are tested with human sera and the results correlated with the results obtained with *D granulomatis* antigen and the same sera.

It will be noted that correlation of negative sera is complete. That is to say, no serum negative with *D granulomatis* antigen gave fixation with any other of

TABLE 1

*Complement-fixation tests using antigens prepared from K rhinoscleromatis, K pneumoniae, E coli, and A aerogenes*

	K RHINO- SCLEROMATIS	K PNEU- MONIAE	E COLI	A AERO- GENES
No. of sera tested	47	68	55	55
Over-all correlation with <i>D granulomatis</i> antigen	94%	78%	69%	64%
Correlation with sera positive with <i>D granulomatis</i> antigen	89%	63%	45%	36%
Correlation with sera negative with <i>D granulomatis</i> antigen	100%	100%	100%	100%

TABLE 2

*Irregular behavior of sera in complement-fixation tests using antigens from D granulomatis, K rhinoscleromatis, K pneumoniae, E coli, and A aerogenes*

SERUM NO.	D GRANULOMATIS	K RHINOSCLERO- MATIS	K PNEUMONIAE	E COLI	A AEROGES
118	+	-	+	-	-
165	+	+	-	+	+
182	+	-	+	-	-

the antigens tested. With sera positive to *D granulomatis* antigen the correlation varied from 89 per cent with *K rhinoscleromatis* antigen to 36 per cent with *A aerogenes* antigen, suggesting a closer relationship to *D granulomatis* to the genus *Klebsiella* than to the other two genera.

If only sera from cases of granuloma inguinale were considered, the positive correlation for *K pneumoniae* antigen rose from 63 per cent to 74 per cent. The figures for the other antigens were not altered.

In general, sera positive with the antigen giving a lower percentage of positive correlation were positive with antigens giving a higher percentage of positive correlation. Thus sera positive with *A aerogenes* antigen were positive with *E coli* antigen, those positive with *E coli* antigen were positive with *K pneumoniae*

antigen. In fact, only three exceptions to this rule occurred (table 2). It will be seen that in two cases there was a negative reaction with *K rhinoscleromatis* antigen and in one with *K pneumoniae* antigen. These negative reactions were out of line in that antigens, which in the over-all picture showed less positive correlation, were positive in these three cases.

#### DISCUSSION

The results of further serological tests presented here demonstrate an antigenic relationship of *D granulomatis* to several members of the tribe *Eschericheae* and support the suggestion made elsewhere (Rake, 1948) that *D granulomatis* should be included in this tribe. If one accepts the degree of correlation in the positive sera as a further indication of the closeness of such relationship, *D granulomatis* is closest to *Klebsiella*, of the three genera tested, and is particularly close to *K rhinoscleromatis* (the presumed etiological agent of rhinoscleroma). The latter fact is of the greatest interest when one recalls the similarities in the etiological problems in the two diseases (Rake, 1948). Other investigators have described common antigens for *K pneumoniae* and *K rhinoscleromatis* (Morris and Julianelle, 1934, Gastings and Sniijders, 1936). We have pointed out elsewhere the morphological similarities between *D granulomatis* and *K pneumoniae* as revealed by the electron microscope (Rake and Oskay, 1948).

#### SUMMARY

Sera fixing complement with an antigen prepared from *Donovania granulomatis* also gave, in a high percentage of instances, fixation with antigens prepared from other members of the tribe *Eschericheae*, namely, *Klebsiella rhinoscleromatis* (89 per cent), *Klebsiella pneumoniae* (63 per cent), *Escherichia coli* (45 per cent), and *Aerobacter aerogenes* (36 per cent).

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## NOTES

### A DEHYDRATED EXPERIMENTAL MEDIUM FOR THE MICROBIOLOGICAL ASSAY OF FOLIC ACID

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This laboratory has employed experimentally a modification of the method described by Teply and Elvehjem in "The titrimetric determination of '*Lactobacillus casei* factor' and 'folic acid' " (J Biol Chem , 157, 303) in which salt solution A and peptone have been omitted from the basal medium and the results of growth are read turbidimetrically after 16 to 18 hours' incubation. Our data indicate that the turbidimetric results are not appreciably affected by the omission of asparagine, alanine, *p*-aminobenzoic acid, peptone, and salt solution B as suggested by Teply and Elvehjem. However, we have omitted only the salt solution A and peptone as a routine procedure. The laboratory preparation of such a medium containing so many constituents requires a considerable amount of time when large volumes are needed at frequent intervals. Also, considerable fluctuation in the quality of this medium has been encountered because of variations in the quality of some of the constituents, particularly the amino acids and the vitamins. Commercial availability of a standardized dehydrated medium that is quickly and easily prepared is highly desirable in a control laboratory where the completion of large volumes of work in short periods of time is important.

Some pantothenic acid assay experiments were performed with a dehydrated "pantothenate assay broth, exp'l" supplied by Difco Laboratories, Inc , Detroit. The protocol supplied with this medium indicated that its composition varied slightly from that described by Skeggs and Wright (J Biol Chem , 156, 21) for the assay of pantothenic acid. The composition of the Skeggs and Wright medium is very similar to that of Teply and Elvehjem. The cardinal difference between the two media is the omission of calcium pantothenate from the former. Other materials present in the folic acid medium but not in the pantothenic acid medium, namely asparagine, alanine, and peptone, are those constituents that Teply and Elvehjem suggested could be omitted.

Previous experimentation with various dehydrated media revealed the presence of a *Streptococcus faecalis* R stimulating substance which enabled the organism to grow in "blank" tubes to which no folic acid aliquots had been added. Irradiation of the liquid medium with ultraviolet light, autoclaving at pH 1.0 for 30 minutes at 15 pounds pressure (121 C), and triple adsorptions with norite A failed to eliminate these stimulating substances without destroying other essential growth factors. However, in the application of the foregoing experimental pantothenate assay medium from Difco to the folic acid assay, it was found that

*S. faecalis* R would grow if calcium pantothenate and folic acid were present. In the absence of folic acid, good blanks without growth were obtained. Maximum growth response to folic acid occurred between the levels of 0.002  $\mu$ g and 0.01  $\mu$ g folic acid aliquots per tube (10 ml) when the optimum concentration of 0.4  $\mu$ g D-Ca-pantothenate per ml were present in the basal medium. Several satisfactory assays were completed using this experimental medium, and the results compared favorably with those obtained by the routine Teply and Elvehjem method. The range of turbidity values between the highest and lowest levels of folic acid concentration, however, was not so great as that usually encountered in our routine assay.

There is evidence to indicate that this range in the turbidity values might be extended by the following changes in the Skeggs and Wright medium before dehydration: (1) the addition of 0.4  $\mu$ g D-Ca-pantothenate per ml basal medium (double strength), (2) the substitution of 25 mg sodium citrate per ml basal medium in place of the sodium acetate, and (3) the addition of 0.1 mg asparagine and 0.2 mg DL-alanine per ml basal medium.

Additional work is in progress to substantiate the value of these suggested changes.

## SALMONELLA CHANDANS: A NEW TYPE ISOLATED FROM A LIZARD

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This new *Salmonella* type was isolated from the intestine of a normal fence lizard (*Sceloporus occidentalis occidentalis*) caught in an abandoned building on a ranch located on Chandans Creek in Napa County, California, by Mr. Jesse Skoss on April 19, 1947. *Salmonella bonarensis* was isolated from the same lizard.

The bacterium has the typical cultural and biochemical characteristics of *Salmonella*. It is a motile, gram-negative rod. Indole was not formed, hydrogen sulfide was produced, and gelatin was not liquefied in 14 days. Acid and gas were produced in 24 hours from arabinose, dulcitol, galactose, glucose, maltose, mannitol, rhamnose, sorbitol, trehalose, and xylose. Acid was produced from glycerol in 2 weeks. Inositol, inulin, lactose, raffinose, salicin, and sucrose were not fermented in 40 days. Sodium citrate and D-tartrate were utilized. Litmus milk was slightly acid in 72 hours, but became alkaline in 1 week.

Antigenic analysis by the method of Edwards and Bruner (Kentucky Agr. Expt. Sta., Circ. 54) showed that the somatic antigen was identical to that of *Salmonella rubislaw*. Alcoholized antigens were agglutinated to titer with both *S. rubislaw* and *Salmonella senegal* antiserums. Absorption of these serums with *S. chandans* removed all the agglutinins for both of these types. Reciprocal



absorption using *S chandans* antiserum removed all the agglutinins for *S chandans*. Therefore the somatic antigen for *S chandans* is XI.

The H antigens were diphasic. Phase 1 was agglutinated to the titers of *Salmonella oregon*, phase 1 (d), and *Salmonella typhi* (d) serums. Phase 2 was agglutinated to the titer of *S rubislaw*, phase 2 (enx) serum, and it was also agglutinated by single factor  $\chi$  serum.

In absorption tests, *S chandans* removed all the H agglutinins from *S typhi* (d) serum and from phase 2 (enx) serum for *S rubislaw*. Reciprocal absorption tests using *S chandans* antiserum verified these results. The antigen formula of *S chandans* is XI d, enx.

#### ACKNOWLEDGMENT

We wish to acknowledge with appreciation the co-operation of Dr. P. R. Edwards, National Salmonella Typing Center, Lexington, Kentucky, in verifying our results.

#### SUMMARY

*Salmonella chandans* (XI d, enx), a new type, was isolated from the intestine of a Pacific fence lizard (*Sceloporus occidentalis occidentalis*), *Salmonella bonariensis* was isolated from the intestine of the same lizard.

### MEDIA PREPARED BY THE PANCREATIC DIGESTION OF MEAT

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For some years we have prepared a basic broth that has given such general satisfaction that we receive frequent inquiries about it. We therefore think that the method of preparation should be placed on record.

To each 250 g of ground, fresh, lean meat (we use beef heart) add 1,000 ml of distilled water and 5 g of sodium chloride. Heat to 50 C and add 2.5 g of pancreatin stirred into a cup of fluid from the above. With occasional stirring, digest at 50 to 56 C for 2 hours. Strain through two layers of fine cotton gauze. Boil for 5 minutes. Filter through coarse filter paper. Restore the volume to 1,000 ml by the addition of distilled water. Adjust the reaction to about pH 7.6. Bring to boiling and filter through paper while hot. Check the reaction and readjust if necessary. Distribute into tubes, flasks, or bottles. Sterilize in the autoclave.

Before this formula was arrived at, we encountered many difficulties such as overdigestion, underdigestion, difficult filtration, turbidity of the final product,

and lack of uniformity The directions, therefore, had better be followed in detail

The meat is only partially digested, and hence the broth contains proteoses, polypeptides, tryptones, and amino acids in amounts slightly greater than are found in a good meat infusion broth prepared from 500 g of meat and 10 g of commercial peptone per liter The medium supports excellent growth of *Brucella*, gonococci, and other fastidious organisms, and seems to serve all the purposes of a good meat infusion broth It may be used as a base for making agar or semisolid media In the form of blood agar it gives typical appearances to the colonies of the various streptococci, including the double zones of those of serological group B

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